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EVALUATION OF BIOAUGMENTATION STRATEGIES TO TREAT HIGH CONCENTRATIONS OF CHLOROFORM AND CARBON TETRACHLORIDE

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EVALUATION OF BIOAUGMENTATION STRATEGIES TO TREAT HIGH CONCENTRATIONS OF CHLOROFORM AND CARBON TETRACHLORIDE

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ABSTRACT

Halogenated methanes, including carbon tetrachloride (CT) and chloroform (CF), are significant groundwater contaminants. Options for bioremediation of high concentrations are limited. Previous studies have shown that an enrichment culture (designated DHM-1) that grows on corn syrup has the potential for use in bioaugmentation. DHM-1 cometabolically biotransforms high concentrations of CT and CF to nontoxic end products (mainly CO, CO_2, and organic acids) in the presence of vitamin B_{12} (approximately 3% of chlorinated methanes on a molar basis). Sulfide is also required by this culture to function properly. However, insufficient data are available on its performance under field conditions. Also, it is not yet known how this culture would perform at low pH levels that may exist in situ, and how the rate of CF biodegradation is influenced by the concentration of B_{12}.

The objectives of this study were 1) to evaluate biostimulation (using corn syrup with and without B_{12}) and bioaugmentation as approaches to treat CT and CF; 2) to evaluate the effectiveness of an enrichment culture (developed during this study) that anaerobically grows on DCM as its sole carbon and energy source, for biodegradation of DCM produced during reaction of CT and CF with zero valent iron (ZVI); 3) to determine the effect of B_{12} concentration on the rate of CF dechlorination by the DHM-1 enrichment culture; and 4) to determine the effect of pH on the rate of CF transformation by the DHM-1 enrichment culture.

To address the first two objectives, microcosms were prepared with subsurface material and groundwater from two industrial sites (Sites A and B) contaminated with high concentrations of CT and CF. To address the other objectives, the DHM-1 culture was subjected to various pH levels and B_{12} concentrations.

The results obtained from the microcosm experiment conducted for Site A indicated that addition of ZVI followed by bioaugmentation was the most promising approach evaluated for removal of CT and CF from the high concentration part of the plume (i.e., in the source zone). This strategy achieved complete removal of CT, nearly complete removal of CF and partial removal of the DCM that accumulated during reaction of the CT and CF with ZVI. There is still a need to determine if the other volatile compounds formed during reaction with ZVI pose a concern for downgradient remediation. Biostimulation with corn syrup plus B_{12} was effective for removal of CT and almost all of the CF from the medium concentration plume. Bioaugmentation did not improve the rate of transformation; however, addition of DHM-1 ensured complete removal of CF. SDC-9 (a commercially available bioaugmentation culture) and a sulfate-reducing enrichment culture (developed for use in this study) were less effective for removal of CT and CF in the medium concentration microcosms.

An anaerobic enrichment culture that grows on DCM as its sole carbon and energy source was successfully developed. It was acclimated to consume up to 500 mg/L of DCM. Use of the culture for bioaugmentation to remove DCM, in Site A microcosms treated with ZVI, was partially effective; additional work is needed to optimize the use of the culture. Although ZVI plus bioaugmentation removed CT and CF at a faster rate, the

accumulation of DCM was a concern that was only partially addressed by bioaugmentation with the DCM enrichment culture.

For the Site A low concentration plume, biostimulation with corn syrup plus B_{12} was the most promising approach evaluated for removal of CT and CF. Adjusting the pH of the groundwater improved the rate of transformation, although this must be weighed against the cost of in situ pH adjustment. No attempt was made to remove the low level of DCM present in the low concentration plume.

The results of the microcosm experiment performed for Site B indicated biostimulation with corn syrup was effective in removing CT; however, addition of B_{12} and bioaugmentation did not appreciably enhance the rate or extent of CT biodegradation. Biostimulation and bioaugmentation did not improve the rate of CF removal over the unamended treatment. The rate of CF removal was slow and consistent in the biotic treatments. None of the amendments were effective in removing DCM, although it was present at a lower concentration than CT and CF. Attempts to stimulate the effectiveness of B_{12} and bioaugmentation by generating sulfide via sulfate reduction were not effective. Although 12 mM of sulfate was consumed in the treatment bioaugmented with DHM-1, there was no enhancement in the rate or extent of CT or CF biodegradation. This suggests that the subsurface material has a considerable sulfide demand that needs to be satisfied before the effectiveness of B_{12} and DHM-1 can be realized.

The results from the experiments conducted to further characterize the DHM-1 enrichment culture indicated that the maximum CF biodegradation rates for DHM-1

increased with increasing pH from 5.0 to 7.7. Its activity is severely inhibited by pH levels below 6.0. At pH 5.0, it lost its ability to biotransform CF. Between pH 6.4 and 7.3, the rate of CF biodegradation by DHM-1 is somewhat stable; however, it increases rapidly between pH 7.3 and 7.7. Given the inhibitory effect of pH levels below 6.0, the pH of the groundwater should be adjusted within the neutral range for successful bioaugmentation to occur. The sensitivity of DHM-1 to low pH values must be considered for application in aquifers that have pH levels below 6. With respect to the dose of B_{12}, CF biodegradation rates for DHM-1 increased with increasing vitamin B_{12} concentrations. The relationship between the B_{12} to CF ratio and the rate of CF biodegradation can be described by a modification of the Michaelis-Menten kinetics model. A V_{max} of 66±4.6 mg CF/L·d and a B_{12}/K_m ratio of 0.0050±0.0010 mol B_{12} per mol CF were obtained from fitting experimental results to this model. This information can help in the selection of a cost-effective dose for B_{12} when it is used as an amendment to facilitate bioremediation of chlorinated methanes.

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LIST OF ABBREVIATIONS

Acronym	Definition
bgs	below ground surface
CF	Chloroform
CM	Chloromethane
CS_2	Carbon Disulfide
CT	Carbon Tetrachloride
1,2-DCA	1,2-Dichloroethane
DCM	Dichloromethane
GC	Gas Chromatograph
MSM	Mineral Salts Medium
MCL	Maximum Contaminant Level
SRB	Sulfate Reducing Bacteria
1, 1,2-TCA	1,1,2-Trichloroethane
ZVI	Zero Valent Iron

1.0 INTRODUCTION AND BACKGROUND

Carbon tetrachloride (CT) and chloroform (CF) are ubiquitous environmental pollutants that are frequently detected in soil and groundwater. Both chemicals are toxic and have similar adverse health effects. According to the Material Safety Data Sheets of these two compounds, chronic exposure to CT or CF can both cause liver and kidney damage and affect the central nervous system. Birth defects may be caused by inhalation or ingestion of CF. And both compounds are group 2B carcinogens, meaning they are "possibly carcinogenic to humans," according to the International Agency for Research on Cancer (27). Their contamination is prevalent because both chemicals were heavily used in industrial applications (8, 19).

CT is a volatile chlorinated solvent which has been used widely over decades as a degreasing agent, as a pesticide, as a precursor to synthesis of freons, for dry cleaning and in fire extinguishers (8). Like CT, CF is also a volatile chlorinated solvent which has a myriad of applications including production of pesticides, extracting pharmaceutical material as a solvent and serving as a precursor in the making of freons. CF also was one of the first surgical anesthetics (19). Use of CT was banned in 1970 in consumer products in the U.S., followed by a complete ban in 1996 by the Montreal protocol (43) due to its deleterious effects on the stratospheric ozone layer and use and emission of CF is also tightly controlled now due to its toxicity (19). Widespread use of CT and CF combined with historically poor disposal practices, spills and leaks from underground storage tanks, and recalcitrance to biodegradation has led to substantial soil and groundwater

contamination. According to the 2007 “Comprehensive Environmental Response, Compensation, and Liability Act Priority List of Hazardous Substances,” CF ranks eleventh overall on this list and is the third highest among chlorinated organics after vinyl chloride and polychlorinated biphenyls. Although not ranked as highly, CT is also listed (1).

Even though the remediation of aquifers contaminated by these pollutants has received much attention, efficient strategies to treat CT and CF are still lacking. With an estimated half-life for abiotic hydrolysis of 7000 years and 1.3-3500 years in water at 20^{o}C, respectively, for CT and CF (46), both compounds are highly persistent in groundwater. When mediated by natural inorganic reductants like Fe(II) or man-made inorganic reductants like zero valent iron (ZVI), CT and CF may undergo rapid abiotic reductive transformation. But when CT or CF are present at high concentrations, less chlorinated but still toxic daughter products like CF and dichloromethane (DCM) are likely to accumulate in large quantities as a result of abiotic processes (10, 31, 42). These daughter products still need to be treated; bioaugmentation with cultures that dechlorinate CF and DCM is an option. However, using ZVI to rapidly initiate the attack on CT is still an attractive remediation strategy since most biological methods appear not to be effective enough to treat high concentrations of CT, (e.g., in the vicinity of a source zone) (10, 32, 42). Successful forms of ZVI application for treatment of chlorinated hydrocarbons include permeable reactive barriers and injection of powder or nano-sized particles. When reacted with CT and CF, ZVI is able to reduce the contaminants to DCM through a reductive dechlorination pathway, or to methane through a reductive

elimination pathway, which is more desirable because of the nontoxic product (10, 42). However, the factors that control the predominant pathway are not yet clear.

Aerobic transformation of CT is unfavorable because its carbon is fully oxidized; microbial CT degradation has only been observed under lower redox conditions. Although aerobic cometabolic transformation of CF is possible, it exerts significant toxicity (44). Thus, bioremediation of these halomethanes, especially at mg per liter concentrations, appears to be limited to anaerobic transformations. However, CT is inhibitory to a variety of anaerobic metabolic processes (7, 12). CF is an even more potent inhibitor (47), especially for reductive dechlorination of chlorinated ethenes (33). When present at high concentrations, CT and CF are not likely to be transformed quickly enough by indigenous microbes due to their inhibitory impacts. Thus, monitored natural attenuation is not likely to be feasible for CT and CF when their concentrations are in the mg per liter range.

So far no organisms capable of using CT as a carbon or an energy source have been isolated. Nor have any organisms that can chlororespire CT as a terminal electron accepter been found, even though the redox potential for the CT/CF couple of +584 mV (9) is very favorable. But cultures and strains capable of CT cometabolic degradation under anaerobic conditions have been reported widely, including sulfate reducing enrichment cultures (15, 39), fermentative enrichment cultures (39) and *Pseudomonas stutzeri* strain KC, which transforms CT under nitrate reducing conditions to innocuous end products without CF accumulation (6). For CF, cometabolic transformation has also been observed under a variety of anaerobic conditions (3, 15, 21-22). More recently, a

Dehalobacter strain was isolated that is able to metabolically halorespire CF to DCM (19). Biostimulation to enhance anaerobic biotransformation of CT and CF has been demonstrated with substrates such as methanol (2), fructose (23) and acetate (37). However, biostimulation typically results in accumulation of lesser chlorinated daughter products like CF and DCM via reductive dechlorination (Figure 1.1). High levels of sulfide favor a substitutive reaction that yields carbon disulfide (CS_2). Although CS_2 is not a regulated compound in drinking water, it is an undesirable endpoint due to its acute toxicity. Addition of cyanocobalamin (vitamin B_{12}) increases the rate of biotransformation and shifts the pathway from reductive dechlorination and CS_2 formation towards hydrolytic and substitutive reactions that yield CO, CO_2, and organic acids (Figure 1.1) (3, 23-24, 39). However, the cost of adding B_{12} is high and the minimum dose required for this cofactor to be effective has not been fully investigated. An alternative to external addition of B_{12} is to grow and harvest B_{12}-producing bacteria (e.g., growing on 1,2-propanediol) and then use these microbes to degrade CT and CF. The ability to produce B_{12} by the biodegrading microbes would be a substantial advantage in terms of cost reduction. Research in this direction has been reported (50), although no reports were found for field scale application.

There are currently no commercial cultures available for bioaugmentation to treat CT and CF. Most of the cultures described in the literature are not applicable to groundwater remediation or the products formed are not acceptable (i.e., carbon disulfide (CS_2) from CT or DCM from CF). In situ biotransformation of CT using *Pseudomonas stutzeri* strain KC is achievable (11) but this microbe needs a high concentration of nitrate

as the terminal electron acceptor, a relatively high pH (~8), and the concentration of CT that it can tolerate is comparatively low, i.e., less than 5 mg/L. The discovery of a *Dehalobacter* strain capable of respiring CF represents an important breakthrough for CF biodegradation (19). However, its end product is DCM; and therefore, another culture that consumes DCM is needed to finish the remediation process.

A fermentative enrichment culture designated DHM-1 which was developed by Shan et al. (40) shows promise for biodegrading CT and CF. This culture cometabolically transforms high concentrations of CT (8.8 mg/L) and CF (500 mg/L CF) in the presence of 3 mol percent of B_{12} (i.e., 0.03 mol of B_{12} per mol of CT or CF). In the presence of B_{12}, the end products are mainly CO, CO_2, and organic acids (39). When provided with corn syrup and B_{12}, DHM-1 is able to grow and biodegrade up to 2000 mg/L of CF in 180 days. It is able to grow on corn syrup at CF concentrations as high as 4000 mg/L (approximately one half its aqueous solubility), although CF was not biodegraded (38). However, a number of characteristics of DHM-1 have not been fully explored, including its ability to function in the field, the extent of its requirement for B_{12}, and the range of pH in which it functions. The main focus of this thesis is to address these issues.

Before evaluating DHM-1 for bioaugmentation in the field, preliminary evaluations were performed in laboratory microcosm studies. For this thesis, samples from two industrial sites were used. To avoid disclosing information about the sites, they were referred to in this thesis as sites A and B. At Site A, the concentration of CT is up to 400 mg/L and the concentration of CF is up to 300 mg/L. At Site B, the concentrations of CT and CF are substantial, but lower than for Site A.

The concentration of CT (400 mg/L) present at Site A is considered to be highly inhibitory to biological processes. Based on concerns that this may be too high for DHM-1, one of the treatments evaluated for this site was addition of ZVI. Preliminary tests indicated that DCM was the main product. Consequently, an additional issue for this site is development of an enrichment culture that biodegrades DCM to nonhazardous products. Unlike CT and CF, DCM is subject to biodegradation as a sole carbon and energy source under anaerobic conditions (13). Cultures that are capable of DCM biodegradation have been reported under fermentative and nitrate reducing conditions, during which DCM is transformed to nonchlorinated products like CO_2 and organic acids (13, 17, 28). Although monitored natural attenuation may be effective for DCM biodegradation at many contaminated sites, bioaugmentation should be considered if the necessary microbes are lacking.

Another finding from preliminary evaluation of groundwater from Site A was the detection of toluene and ethylbenzene at low concentrations (~1 mg/L), along with CT and CF. Under anaerobic condition, metabolic degradation of toluene and ethylbenzene has been well demonstrated (5, 29, 36). Ethylbenzene can be degraded under nitrate reducing, sulfate reducing, and fermentative conditions. Compared to ethylbenzene, toluene can undergo transformation even faster under a broader range of terminal electron accepting conditions. Both compounds serve as growth substrates under anaerobic conditions. Therefore, complete biodegradation of toluene and ethylbenzene was expected after removing the more toxic CT and CF.

2.0 RESEARCH OBJECTIVES

The overall objective of this thesis was to evaluate strategies to remediate groundwater contaminated with high concentrations of CT and CF, with a focus mainly of further characterizing the DHM-1 enrichment culture developed by Shan et al. (40). The specific objectives were:

1) To evaluate biostimulation (using corn syrup with and without B_{12}) and bioaugmentation as approaches to treat CT and CF. Three types of bioaugmentation cultures were evaluated for this purpose:
    - DHM-1, for biodegradation of high concentrations of CT and CF;
    - SDC-9, a commercially available bioaugmentation culture that exhibits activity on CT and CF; and
    - An enrichment culture of sulfate reducing bacteria (SRB), developed over the course of this research, for biodegradation of CT and CF
2) To determine the effect of B_{12} concentration on the rate of CF dechlorination by the DHM-1 enrichment culture;
3) To determine the effect of pH on the rate of CF transformation by the DHM-1 enrichment culture; and
4) To evaluate the effectiveness of an enrichment culture that anaerobically grows on DCM as its sole carbon and energy source, developed over the

course of this research, for biodegradation of DCM produced during reaction of CT and CF with ZVI.

3.0 MATERIALS AND METHODS

3.1 Chemicals

CT (99.9%, Sigma–Aldrich), CF (99.7%, Shelton Scientific), DCM (99.9%, AlliedSignal), 1,1-dichloroethene (99%, TCI America), *cis*-1,2-dichloroethene (99%, TCI America), 1,2-dichloroethane (1,2-DCA; 100%, Mallinckrodt), 1,1,2-trichloroethane (1,1,2-TCA; 98%, Acros Organics), CS_2 (100%, J.T. Baker), benzene (99%, Sigma–Aldrich), toluene (99%, Fisher Scientific), and ethylbenzene (99%, Alfa Aesar) were obtained as neat liquids. Chloromethane (CM; 99%, Praxair), ethene (99.95%, Airgas), ethane (99%, Matheson), and methane (99.99%, Matheson) were obtained as neat gases. 1,1-dichloroethane standard (2000 µg/mL, VWR) was obtained as methanol solution. Sodium lactate (60% syrup, EM Science) and corn syrup (regular type, Sweetener Products Company) were used as electron donors. The chemical oxygen demand for corn syrup and sodium lactate was calculated based on their presumed composition, i.e., $C_6H_{12}O_6$ and $C_{18}H_{32}O_2$, respectively. Cyanocobalamin (i.e., vitamin B_{12}, USP grade) was obtained from Research Organics, Inc. Micron scale high reactivity ZVI powder was obtained from Hepure Technologies, Inc. All other reagents were ACS grade or higher.

3.2 Analytical Methods

Halogenated (CT, CF, DCM, CM, 1,1-dichloroethene, 1,2-DCA, and 1,1,2-TCA) and nonhalogenated volatile compounds (methane, ethane, ethene, CS_2, benzene, ethyl benzene, and toluene) were monitored by gas chromatographic analysis of 0.5 mL

headspace samples (14). A Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector and a 2.44 m × 3.175 mm column packed with 1% SP-1000 on 60/80 Carbopack B (Supelco) was used. A GC response factor for each volatile organic compound was measured to relate the total mass of the compound to the GC peak area. Results were reported in terms of the μmoles per bottle, in order to easily reveal the stoichiometry of the transformations (e.g., moles of CF formed per mole of CT transformed). Aqueous phase concentrations are also reported based on partitioning of the compounds between the headspace and aqueous phases, as previously described (45). The detection limit of this method is lower than the maximum contaminant level (MCL) for each of the chlorinated compounds. Representative response factors for each compound are provided in Appendix A.

Organic acids (acetate, propionate and lactate) were analyzed on a Waters 600E HPLC system equipped with a UV/Vis detector (Model 490E) set at 210 nm and an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm; BioRad). Eluant (0.01N H_2SO_4) was delivered at 0.6 mL per min. Representative response factors for each compound are provided in Appendix B. Samples were removed from microcosms by setting them in an upright position, allowing the solids to settle, and then using a glass syringe (1 mL) with a long needle to remove 1 mL of the clarified groundwater. Then 0.5 mL of the removed groundwater was filtered and discarded using a syringe filter (0.2 μm, Whatman) to condition the filter. The other 0.5 mL was then filtered and stored in a HPLC sample vial until analysis.

Sulfate was measured on a Dionex DX-600 ion chromatography system equipped with a CD25 Conductivity Detector and a Dionex guard column (AG9-HC, 4 mm x 50 mm) followed by an IonPac AS9-HC anion-exchange column (4 mm x 250 mm). Eluant (9 mM Na_2CO_3) was delivered at 1.0 mL per min. A representative response factor is provided in Appendix C. Samples from the microcosms were taken and processed in the same manner described above for organic acids.

3.3 Enrichment Cultures

Five enrichment cultures were evaluated in the course of this research. A description of each one follows.

3.3.1 DHM-1 Enrichment Culture

The DHM-1 enrichment culture developed by Shan (40) was maintained for use in this thesis. The enrichment was maintained in two 160 mL serum bottles with 100 mL of mineral salts medium (MSM) (the components of the media are shown in Table 3.1) plus corn syrup as the electron donor (5 mM), B_{12} as the catalyst (136 µM) and CF (36 µL neat = 452 µmol per bottle, or an aqueous phase concentration of 500 mg/L). For routine purposes, the molar ratio of B_{12} to CF was 3%. The headspace of the serum bottles was purged with a gas mixture (30% CO_2/70% N_2) and the serum bottles sealed with Teflon-faced red rubber septa and aluminum crimp caps. Headspace analysis by GC was conducted every four to five days. When the remaining CF was below 0.5 mg/L, the culture was transferred to fresh medium (5% v/v) along with a new dose of CF, B_{12} and corn syrup. It was expected that approximately one month or less was needed to

biodegrade 500 mg/L of CF. The typical behavior of the DHM-1 enrichment culture is shown in Figure 3.1.

3.3.2 SRB Enrichment Culture

A lactate-grown SRB enrichment culture was started in a 250 mL glass bottle containing 200 mL of MSM (Table 3.1), 37.1 mM sulfate, 3 mM sodium lactate and 20 g of subsurface material from the high concentration plume of Site A (described below). Growth was monitored based on consumption of lactate and production of acetate. When the lactate was consumed, more was added. The maximum dose of lactate was kept at 2 mM to avoid enriching for a fermentative culture that converts lactate to propionate plus acetate, rather than a SRB culture. Once a consistent level of lactate consumption and acetate production was demonstrated, the enrichment was transferred (1% v/v) to MSM after consuming approximately 10 mM lactate without significant propionate formation; the culture was then considered ready for use in bioaugmentation. The performance of the SRB enrichment culture is shown in Figure 3.2.

3.3.3 DCM Enrichment Culture

An enrichment culture that anaerobically consumes DCM as its sole energy and carbon source was developed from a previous microcosm study conducted at Clemson University. DCM at a concentration of 13-15 mg/L was biodegraded in the microcosms without accumulation of lesser chlorinated products. DCM biodegradation in the microcosms was enriched by gradually increasing the dose to 500 mg/L. An aliquot from the microcosm was then transferred to an anaerobic MSM (Table 3.1) (1% v/v) to begin development of a sediment-free enrichment culture. The culture was used for

bioaugmentation of microcosms in which DCM accumulated. The behavior of the DCM enrichment culture is shown in Figure 3.3.

3.3.4 SDC-9 Enrichment Culture

A commercially available enrichment bioaugmentation culture (SDC-9) was obtained from Dr. Rob Hinchee at Shaw Environmental, Inc.. The culture was used to bioaugment selected microcosms immediately upon receipt.

3.3.5 1,2-DCA Enrichment Culture

A dehalorespiring enrichment culture that uses 1,2-DCA as its terminal electron acceptor and lactate as its electron donor was added to treatments that received ZVI (Table 3.2), based on the presumption that 1,2-DCA had formed during reaction of the chlorinated compounds with the chlorinated methanes. The culture converts 1,2-DCA to ethene via dihaloelimination and organohalide respiration; the reaction is carried out by *Dehalococcoides* (34, 47). Additional characteristics of the 1,2-DCA enrichment culture are described by Yu (48) and Peethambaram (34).

3.4 Experimental Design for Microcosms

The experimental designs used to evaluate bioremediation strategies for Sites A and B are described below. In each case, microcosms were used for the evaluation.

3.4.1 Site A

The potential for bioremediation of groundwater contaminated with high concentrations of CT and CF at Site A was evaluated using the experimental design outlined in Table 3.2 and described below. Along with chlorinated methanes, the groundwater also contains acetone, 1,1-dichloroethene, 1,1-dichloroethane, *cis*-1,2-

dichloroethene, benzene, toluene and ethyl benzene. The peak areas for toluene and ethylbenzene were one order of magnitude higher than the other compounds and, therefore, they were quantified. For the other compounds, their GC peak areas were recorded but they were not quantified.

The study consisted of three sets of serum bottles targeting three different levels of CT and CF concentrations observed at different locations (Table 3.3) on the site:

1) High concentration plume (source zone) (CT ~400 mg/L, CF ~340 mg/L);
2) Medium concentration plume (CT ~50 mg/L, ~CF 20 mg/L); and
3) Low concentration plume (CT ~5 mg/L, CF ~1-2 mg/L).

Treatments for the high concentration plume included unamended controls (H-Con); biostimulation with corn syrup (H-BST); biostimulation with corn syrup plus vitamin B_{12} (H-BB12); bioaugmentation with DHM-1 plus B_{12} and corn syrup (H-BIOA); bioaugmentation with a commercial bioaugmentation culture (SDC-9) plus B_{12} and lactate (H-BIOB); bioaugmentation with the sulfate-reducing enrichment culture plus B_{12} and lactate (H-BIOC); ZVI followed by bioaugmentation with a DCM-degrading enrichment culture (H-ZBIO); autoclaved controls (H-AC); and water controls (H-WC). Treatments for the medium concentration plume were the same, but without water controls (the higher concentration water controls were considered sufficient to evaluate diffusive losses). The treatment identifiers were modified by replacing the leading "H" with "M" (e.g., H-Con was changed to M-Con).

Treatments for the low concentration plume control included unamended controls (L-Con); biostimulation with corn syrup (L-BST); biostimulation with corn syrup plus

vitamin B_{12} (L-BB12); biostimulation with corn syrup plus vitamin B_{12} along with pH adjustment (L-ABST); and autoclaved controls (L-AC). It was anticipated that bioaugmentation would not be needed for the low concentration microcosms.

All treatments were prepared in triplicate in an anaerobic chamber. All treatments except the water controls received subsurface material (composited from multiple core samples at varying depths) and groundwater collected from the three locations. The water control bottles received distilled deionized water and CT, CF, and DCM in approximately the same amounts as in the high concentration groundwater; glass beads were added to displace the same volume of water (~11 mL) as the subsurface material in the other treatments.

3.4.2 Site B

Microcosms were also used to assess bioremediation of subsurface material and groundwater from Site B, which contains approximately 20 mg/L of CT and 80 mg/L of CF. The experimental design for the microcosm experiments for Site B is summarized in Table 3.4 and described below, for the following treatments: Unamended (Con); biostimulation (BST) with corn syrup; biostimulation with corn syrup + B_{12} (BB12); bioaugmentation with DHM-1 (BIO) (which includes addition of corn syrup + B_{12}); and autoclaved controls (AC). All treatments were prepared in triplicate.

3.5 Microcosm Preparation

3.5.1 Site A

Subsurface material and groundwater were collected from three locations representing the high (source zone), medium and low concentration plumes. For the high

concentration part of the plume, subsurface material was collected from the bottom of the saprolite zone from a boring near piezometer PZ-95 (approximately 8-10 feet below ground surface (bgs)). For the medium concentration part of the plume, subsurface material was collected from the bottom of the saprolite zone at monitoring well TP-1 (approximately 35-37 feet bgs). For the low concentration part of the plume, subsurface material was collected from the bottom of the saprolite zone from a boring near piezometer PZ-12 (approximately 45-47 feet bgs). The subsurface material from TP-1 and PZ-12 was collected with 2.5 foot-long Shelby tubes which were filled with about 1 kg of subsurface material and sealed with wax on both ends. The subsurface material from PZ-95 was collected and sealed using a clean bucket from 8-10 feet bgs since the density of the subsurface material from this area would crush the Shelby tube, but air exposure was minimized. All subsurface material was shipped on ice to Clemson University.

Groundwater samples were collected from piezometer PZ-95-2 (47-52 feet bgs) for the high concentration part of the plume, from TP-4 monitoring well (30-40 feet bgs) for the medium concentration part of the plume, and from piezometer PZ-12 (49- 54 feet bgs) for the low concentration part of the plume. Samples were shipped to Clemson University in brown glass 1 L bottles, on ice, via an overnight carrier, within 24 hours of taking the samples. Samples were stored in a cold room until the microcosms were prepared.

Subsurface material was removed from the Shelby tubes before microcosm preparation by using a power saw to cut off a portion of the tube which was filled with

wax, so that the wax layer would not contaminate the subsurface material sample. All subsurface material was transferred to three containers sterilized with ethanol and stored in an anaerobic chamber. Before setting up the microcosms, groundwater samples were transferred to the anaerobic chamber and allowed to warm to room temperature.

Prior to setting up the microcosms, groundwater samples from each plume were evaluated by GC in duplicate in serum bottles containing 50 mL of groundwater and 11 mL of glass beads (the same volume as used in water controls). Results from this evaluation showed that CF was similar to the field conditions (Table 3.3): 246±40, 27.1±0.2, and 0.28±0.01 mg/L for groundwater from the high, medium and low concentration plumes, respectively. Consequently, no changes were made to the groundwater with respect to CF. However, the CT levels were considerably lower than expected (Table 3.3): 36.3±12.9, 22.0±0.02, and 1.38±0.02 for groundwater from the high, medium and low concentration plumes, respectively. Consequently, CT was added to the groundwater, either as a saturated water solution (~5.2 µmol/mL) or as a neat compound. The decision of which to add depended on the volume; the lowest volume of neat CT added was 3 µL, or 10.4 µmol/bottle; if the amount required was less than that, the water-saturated solution was used. Sufficient CT was added so that the initial microcosm concentrations were similar to the field levels (Table 3.3).

The pH of the groundwater samples was checked before setting up the microcosms. The groundwater from the high and medium concentration plumes was within the desired range of 6.5-7.5. However, as corn syrup was fermented to organic acids, the pH decreased. To keep the pH in the range of 6.5 to 7.5, NaOH (8 M) was

added periodically. The groundwater from the low concentration plume was below 6, which is often unfavorable for bioremediation. The pH in the L-ABST treatment (Table 3.2) was adjusted to 6.6-7.5 using NaOH (8 M), and was maintained in this range throughout the incubation period.

The amount of electron donor added to the appropriate treatments, in the form of corn syrup or lactate (Table 3.2), was based on supplying 500 mg/L of chemical oxygen demand in excess of the amount needed to reduce sulfate in the groundwater to sulfide. The initial concentration of sulfate was 0.89, 3.45 and 0.18 mM, in groundwater from the high, medium and low concentration plumes, respectively. For example, for the high concentration microcosms, 77 µL of a corn syrup stock solution containing 360 mg/mL of chemical oxygen demand was added, which provided 557 mg/L of chemical oxygen demand (or 27.9 mg/bottle).

Microcosms were prepared in 160 mL serum bottles by adding 20.0±0.2 g of subsurface material and 50±1 mL of groundwater, inside an anaerobic chamber. The bottles were then sealed with a Teflon-faced red rubber septum and aluminum crimp cap. Resazurin was added to the groundwater to achieve a concentration of 1 mg/L and serve as a redox indicator. Within three days of preparing the microcosms and allowing the volatile compounds to equilibrate, the treatments with ZVI were returned to the anaerobic chamber, and were opened briefly while 0.2 g of ZVI was added per bottle to achieve a one percent weight ratio of ZVI to subsurface material; they were immediately resealed and removed from the chamber. The microcosms were then placed on a shaker table for

24 h to facilitate obtaining equilibrium between the subsurface material and liquid phase prior to taking headspace samples for analysis of the volatile compounds.

The amount of vitamin B_{12} added was based on a stepwise strategy, since the degradation of CT and CF was expected to occur in a stepwise manner (i.e., CT first, then CF). Therefore, the first dose of B_{12} was based on the initial amount of CT and the second dose was based on the remaining amount of CF. Both doses were kept at a molar ratio of 3% B_{12} to CT or CF.

The first bioaugmentation dose and first B_{12} addition were made after electron donor had been added into each bottle and low redox conditions had developed, based on a color change in the resazurin from pink to clear. For DHM-1, SDC-9, and the SRB enrichment cultures, the volume of the addition was adjusted so that the resulting protein concentration in the microcosms was 5 µg/mL: 0.50 mL/bottle for DHM-1, 0.13 mL/bottle for SDC-9, and 1.0 mL/bottle for the SRB culture. A second dose of the bioaugmentation culture (the same volumes listed above) was made once CT was consumed and was accompanied by the second dose of B_{12}. For ZVI treatments that yielded high amounts of DCM, bioaugmentation with the DCM enrichment culture was performed (0.5-1.0 mL/bottle). Bioaugmentation with SDC-9 or DHM-1 was used to facilitate the removal of remaining CF, prior to adding the DCM enrichment culture. The 1,2-DCA enrichment culture was added only to the H-ZBIO and M-ZBIO treatments (0.50 mL/bottle).

3.5.2 Site B

For Site B, subsurface material was collected from location S-1464-MW08-03S and groundwater samples from monitoring well MW-R06-03. Samples were stored at 4°C prior to preparing the microcosms.

Microcosms were set up in the same manner as for Site A, as described above. For Site B, however, there was not a ZVI treatment since the concentrations of CF and CT at this site were low enough that bioremediation alone was anticipated to be successful. Only one enrichment culture, DHM-1, was tested for the bioaugmentation treatment. A dose of 0.50 mL/bottle was used to achieve a protein concentration of 5 µg/mL. Two more doses of bioaugmentation were made when observations suggested that the first dose did not facilitate transformation of CT and CF. Subsurface material and groundwater were collected from only one location at this site.

Because most of the contaminant mass (CT, CF, DCM, 1,2-DCA and 1,1,2-TCA) was destroyed after the AC microcosms were autoclaved, it was necessary to add these compounds as neat liquid or saturated water solution to restore the initial concentrations to a similar level as in the other treatments. The specific quantities added were: 2 µL of CT as neat compound; 335 µL of CF saturated water (~67 µmol/mL); 2 µL of DCM saturated water (~235.5 µmol/mL); 29 µL of 1,2-DCA saturated water (~87.8 µmol/mL); and 43 µL of 1,1,2-TCA saturated water (~33.7 µmol/mL).

3.6 Evaluating the Effect of B_{12} Concentration on CF Biodegradation by DHM-1

The effect of vitamin B_{12} concentration on CF transformation rates by DHM-1 was evaluated in serum bottles as described in Section 3.3.1, except that the concentration

of B_{12} was varied from 0.000 to 0.030 mol B_{12} per mol of CF added (500 mg CF/L = 4.19 mM), only one dose of corn syrup was added, and the bottles were continuously mixed on a shaker table. Media controls were included to evaluate abiotic losses of CF. The highest CF biodegradation rate for a given B_{12} dose was determined by linear regression of CF concentration versus time. The results for all B_{12} doses were fit (using Matlab, version 7.10.0) to a modified form of the Michaelis-Menten model:

$$V = \frac{V_{max} \cdot \frac{B_{12}}{C_o}}{\frac{B_{12}}{K_M} + \frac{B_{12}}{C_o}} \tag{3.1}$$

where V = rate of CF biodegradation (mg/L·d); V_{max} = maximum rate of CF biodegradation (mg/L·d); B_{12}/C_o = the amount of B_{12} added (µmol/bottle) divided by the amount of CF added (µmol/bottle); and B_{12}/K_M = molar ratio at which V is one half of V_{max}.

3.7 Evaluating the Effect of pH on CF Biodegradation by DHM-1

Biodegradation rates for 500 mg/L of CF by DHM-1 were measured at pH levels from 5.0 to 7.7. B_{12} (0.030 mol per mol of CF added) and corn syrup (900 mg/L) were added to all treatments. Serum bottles were prepared as described above for the B_{12} dose experiment, with the following modifications. The MSM was prepared at the target pH by varying the amounts of K_2HPO_4 and KH_2PO_4 (Appendix D). After adding sodium sulfide, the final pH was adjusted using either H_3PO_4 (1 M) or NaOH (8 M); the MSM was incubated for six days to ensure equilibrium was reached at the target pH, before inoculating the DHM-1 enrichment culture (5% v/v). It was not necessary to sparge the

headspace of the bottles with 30% CO_2/70%. Each time CF was analyzed on the GC, the pH was measured (0.2 mL sample) and, as needed, increased to the target level using NaOH (8 M); decreases in pH were caused by fermentation of the corn syrup to organic acids. The highest CF biodegradation rate at a given pH was determined in the same manner described above for varying B_{12} doses. Lag times were based on the time from day zero to the first data point used to determine the highest biodegradation rate.

4.0 RESULTS

4.1 Site A

4.1.1 High Concentration Plume (CT ≈ 500 mg/L, CF ≈ 250 mg/L)

Results for the AC and WC treatments are shown in Figures 4.1 and 4.2, respectively. Percent decreases are summarized in Table 4.1. Over an incubation period of 577 days, the losses in the autoclaved controls for the C_1 compounds (CT, CF, DCM and CS_2) ranged from 24 to 29%, while losses for toluene (52%), and ethylbenzene (59%) were approximately twice as high. In the water controls, CT decreased by 28%, CF by 36%, and DCM by 21%, most likely via diffusion. Overall, these results indicate that losses from the abiotic controls were acceptable given the long incubation time, when compared to the magnitude of losses in several of the active treatments (described below).

The unamended treatment (Figure 4.3) behaved similarly to the AC bottles (Figure 4.1), with similar average losses for CT, CF, DCM, CS_2, ethylbenzene, and toluene (Table 4.1). This indicated that there was no significant biotic activity in the unamended treatment (Student's t-test, α=0.05), which is consistent with the lack of activity observed in the field.

As shown in Figure 4.4 and Table 4.1, biostimulation with corn syrup alone did not significantly improve the removal of CT, CF, DCM, CS_2, ethylbenzene, and toluene over the unamended controls (Student's t-test, α=0.05). Adding B_{12} along with corn syrup also did not significantly improve the rate or extent of halomethane biodegradation

(Figure 4.5; Table 4.1; Student's t-test, α=0.05). The first dose of B_{12} was not made until day 67, to allow sufficient time for low redox conditions to become established in the microcosms. This was based on a change in color of the resazurin added to the groundwater from pink to clear, indicating that the E_h level decreased below -110 mV (a level more conducive to anaerobic dehalogenation). The dose added corresponded to 3 mole percent of the CT initially present (458 µmol/bottle). A second dose of B_{12} (the same amount as the first) was made on day 554; however, the next sampling point indicated no apparent improvement in the average level of CT or CF removal. It should be noted that CT decreased noticeably in one of the three bottles (#3) after the second dose of B_{12}, without an increase in CF. However, the lack of consistent performance among the replicates suggests that adding corn syrup + B_{12} will not be a reliable treatment approach for the high concentration plume.

Bioaugmentation was evaluated with three enrichment cultures. Table 4.1 indicates only a modest level of improvement in the average extent of CT removal for the treatment with DHM-1 added. Most of this increase in percent removal was attributable to a single bottle; as shown in Figure 4.6 for bottle #3, CT decreased at an accelerating rate from 452 µmol/bottle (432 mg/L) on day 245 to 0.1 µmol/bottle (0.1 mg/L) on day 570, without a commensurate increase in CF. A second addition of B_{12} appeared to yield a modest decrease in CF, although additional incubation time would have been needed to determine if this impact was sustainable. Regardless, the lack of consistent results for CT and CF among the replicates casts doubt on the effectiveness of this treatment strategy for

the high concentration plume. Results for the other two bottles are provided in Appendix E.

Bioaugmentation with SDC-9 was also ineffective. As shown in Table 4.1, the average percent removal of halomethanes from this treatment was not significantly different from the autoclaved controls or unamended treatment (Student's t-test, α=0.05). As indicated in Figure 4.7, lactate was used as the electron donor for this treatment, and B_{12} was added at approximately the same time as the addition of SDC-9. Results for the bioaugmentation treatment employing the SRB culture were similar, with no significant increase in CT or CF removal in comparison to the autoclaved and unamended controls (Table 4.1; Student's t-test, α=0.05). Lactate was used as the electron donor for this treatment, as indicated in Figure 4.8.

In contrast to the other treatments, addition of ZVI (0.2 g/bottle) resulted in complete removal of CT and nearly complete removal of CF (Table 4.1). Representative results for one of the microcosms (bottle #1) are shown in Figure 4.9; results for the other two bottles are shown in Appendix E. CT transformation started immediately after the addition of ZVI and decreased from 469 µmol/bottle (448 mg/L) to 124 µmol/bottle (118 mg/L) on day 2, followed by a slower rate of removal until CT was below detection by day 46. There was a corresponding increase in CF, from 136 µmol/bottle (258 mg/L) on day 0 to 310 µmol/bottle (585 mg/L) on day 2. Then transformation of CF started, decreasing to 9.1 µmol/bottle (17 mg/L) on day 59, with relatively little further decrease thereafter. By day 59, 54% of the initial CT and CF had been converted to DCM (on a molar basis). Over a similar time interval, there was a notable increase in another

compound, presumptively identified (based on coelution with authentic material) as 1,2-DCA. In addition, at least ten other volatile compounds appeared in the chromatograms following the addition of ZVI (examples are shown in Appendix F); identification of these peaks will require further evaluation.

Although most of the CF was removed within two months of adding the ZVI, a significant amount persisted (17 mg/L). The first attempt to further decrease the CF was made by adding lactate on day 7, followed by addition of B_{12} (14.1 µmol, to achieve a 3% molar ratio to the initial CT amount per bottle) and bioaugmentation with SDC-9 on day 66; this did not yield an appreciable decrease in CF. The second attempt was made on day 270 by adding DHM-1 and a second dose of B_{12} (0.15 µmol/bottle, based on a 3% molar ratio to the remaining CF); however, no further decrease in CF concentration was observed.

To address the high level of DCM that accumulated, the microcosms were bioaugmented on day 214 with the DCM enrichment culture, which uses DCM as its sole carbon and energy source. And after a lag period of 94 days, transformation of DCM started and the level decreased from 304 µmol/bottle (446 mg/L) to 30 µmol/bottle (45 mg/L) on day 474, but further decreases did not occur. On day 531, another dose of DCM culture was added but this did not revive the prior DCM biodegradation activity; it remains unclear why the significant level of DCM biodegradation did not continue until the compound was completely removed.

To address the accumulation of the presumptively identified 1,2-DCA, the microcosms were bioaugmented on day 251 with a 1,2-DCA respiring culture, along with

lactate, which the culture uses as its electron donor. However, there was no subsequent decrease in 1,2-DCA. The persistence of the CF may have negatively impacted the activity of this enrichment culture, which is dominated by *Dehalococcoides*.

The average performance of each treatment with respect to CT, CF and DCM is summarized in Figure 4.10. As previously described, the treatment with ZVI was the most effective with respect to CT and CF but it resulted in a large increase in DCM. This was partially addressed by bioaugmenting the ZVI bottles with a DCM-degrading anaerobic enrichment culture, although complete removal of the DCM was not achieved. Among the biotic-only treatments, bioaugmentation with DHM-1 showed the most promise, although this was largely due to activity in a single bottle, while the other two showed comparatively little activity. Taken together, these results suggest that addition of ZVI may be a workable strategy for the highest concentration area of the contaminant plume, presuming that the daughter products formed (DCM and a number of other, as yet undefined compounds) can be removed as they migrate downgradient. Further testing would be needed to evaluate this.

4.1.2 Medium Concentration Plume (CT ≈ 70 mg/L, CF ≈ 24 mg/L)

Results for the AC treatment are shown in Figure 4.11. Percent decreases are summarized in Table 4.1. Over an incubation period of 609 days, losses in the autoclaved controls for the C_1 compounds (CT, CF, DCM and CS_2) ranged from 14 to 23%. The loss for toluene was 43% and ethylbenzene was not present in most of the treatments for the medium concentration. Overall, these results are consistent with those from the autoclaved controls for the high concentration plume; abiotic losses were generally

insignificant (Student's t-test, α=0.05) in comparison to the biotic and ZVI treatments (described below).

The unamended treatment (Figure 4.12) behaved similarly to the AC bottles (Figure 4.11); decreases in CT, CF, DCM, CS_2, and toluene were somewhat higher than for the AC treatment, but with overlapping standard deviations (Table 4.1). This indicated that there was no significant biotic activity in the unamended treatment (Student's t-test, α=0.05), which is consistent with the lack of activity observed in the field.

Biostimulation with corn syrup alone resulted in a nearly 96% decrease in CT (Table 4.1) over an incubation period of 609 days. As shown in Figure 4.13, CT decreased from 71 µmol/bottle (68 mg/L) to 3 µmol/bottle (3 mg/L); corn syrup was added five times. The decrease in CT was accompanied by a 25 µmol/bottle increase in CF (from 12 µmol/bottle (23 mg/L) to 37 µmol/bottle (70 mg/L)). There was no significant change in DCM, CS_2, or toluene in comparison to the unamended control treatment (Student's t-test, α=0.05). These results are consistent with expectations that addition of electron donor alone may stimulate reductive dechlorination of CT, accompanied by a significant (but not stoichiometric) increase in CF.

Biostimulation with corn syrup plus B_{12} achieved complete transformation of CT and nearly complete removal of CF (Table 4.1). The performance of a representative microcosm (bottle #2) for this treatment is shown in Figure 4.14; results for the other two bottles are provided in Appendix E. In this microcosm, B_{12} was added on days 66 and 198. After the first dose of B_{12} and second dose of corn syrup, biotransformation of CT

started approximately seven days later, decreasing from 62 µmol/bottle (60 mg/L) to 0.4 µmol/bottle (0.4 mg/L) by day 216; by day 392, CT decreased below its MCL of 0.005 mg/L. There was no increase in CF as CT was biotransformed. Instead, around day 306, CF decreased from 12 µmol/bottle (23 mg/L) to below its MCL of 0.080 mg/L by day 392. These results clearly established that adding B_{12} resulted in complete biodegradation of CT and CF in the medium concentration plume, with MCLs reached within approximately 400 days. No significant formation or decrease in DCM, CM, CS_2, or toluene was observed in comparison to the unamended controls (Student's t-test, α=0.05).

Biostimulation with corn syrup plus B_{12} combined with DHM-1 bioaugmentation also resulted in complete removal of CT and CF (Table 4.1). However, as shown for a representative microcosm (bottle #2; results for the other two bottles are provided in Appendix E), the rate of CT and CF transformation was faster than with corn syrup plus B_{12} addition alone (Figure 4.15). Corn syrup and B_{12} were added at the same times and amounts as for the corn syrup plus B_{12} treatment; DHM-1 was added on days 66 and 243. CT decreased from 61 µmol/bottle (59 mg/L) to 0.6 µmol/bottle (0.6 mg/L) on day 210 and dropped below the MCL on day 342, approximately 50 days earlier than biostimulation with corn syrup plus B_{12}. Close to day 306, CF transformation started and dropped below the MCL before day 428. In an attempt to remove the DCM, the DCM enrichment culture was added on day 520. DCM decreased from 12 µmol/bottle (17 mg/L) to 9 µmol/bottle (13 mg/L) by day 599; additional incubation would be required to determine if this trend was sustainable. No significant formation or decrease in CM, CS_2,

or toluene was observed in comparison to the unamended controls (Student's t-test, α=0.05). It is notable that in this treatment, methane began to accumulate after day 520. This indirectly reflects the removal of CT and CF, which are potent inhibitors of methanogenesis.

Biostimulation with lactate plus B_{12} combined with SDC-9 bioaugmentation resulted in complete removal of CT and 88% removal of CF (Table 4.1). Two of the three microcosms achieved complete transformation of CF over an incubation period of 599 days. In the third microcosm, only 64% removal of CF was observed at the end of the incubation period; a second dose of B_{12} was added on day 554, yet 45 days later the CF still persisted. In the representative microcosm (Figure 4.16; results for the other two bottles are provided in Appendix E), B_{12} was added on day 66 and SDC-9 was added on days 66 and 347; the lack of a second addition of B_{12} (once the CT was consumed) was an oversight. This was fortuitous, however, since the results showed that complete CF removal was possible without the second B_{12} addition in two of the three microcosms. In the representative microcosm (Figure 4.16), CT decreased from 75 µmol/bottle (71 mg/L) to 0.24 µmol/bottle (0.23 mg/L) on day 247 and dropped below the MCL on day 528. Close to day 247, CF transformation started but the rate was relatively slow. After the second dose of SDC-9, the CF transformation rate increased substantially and transformation below the MCL was complete by day 528. In an attempt to remove DCM, the DCM enrichment culture was added on day 528; however, there was no apparent response through day 599, when monitoring ended. No significant formation or decrease

in CM, CS_2, or toluene was observed in comparison to the unamended controls (Student's t-test, α=0.05).

Biostimulation with lactate plus B_{12} combined with bioaugmentation using the SRB enrichment culture resulted in 94% removal of CT but no removal of CF (Table 4.1). Over an incubation time of 599 days, two of the three microcosms completed transformation of CT to below its MCL; in the third bottle, only 83% of the CT was transformed. Figure 4.17 shows the results for bottle #1; results for the other two bottles are provided in Appendix E. B_{12} and SRB culture were added on day 170. CT decreased to below its MCL by day 486, which was a notably slower rate than in the other biotic treatments that also received B_{12}. CF did not increase during this interval, nor did it decrease significantly during the remainder of the incubation period. A second dose of bioaugmentation culture was not made, since the SRB culture was not maintained over this extended interval. While it is possible that a second dose of SRB culture may have resulted in CF removal, the slower rate of activity on CT suggests the SRB culture is not an effective alternative to DHM-1 or SDC-9.

Addition of ZVI (0.2 g/bottle) resulted in complete removal of CT and CF (Table 4.1). Representative results for one of the microcosms (bottle #2) are shown in Figure 4.18; results for the other two bottles are provided in Appendix E. The patterns observed were similar to those for the high concentration plume (Figure 4.9). CT transformation started immediately, decreasing from 70 µmol/bottle (67 mg/L) to 1.2 µmol/bottle (1.2 mg/L) on day 2 and below the MCL by day 33. CF initially increased from 13 µmol/bottle (24 mg/L) to 36 µmol/bottle (69 mg/L), then decreased to below the MCL by

day 66. While CT and CF were undergoing transformation, DCM increased from 14 µmol/bottle (20 mg/L) to a peak of 76 µmol/bottle (111 mg/L). The daughter product presumptively identified as 1,2-DCA appeared in this treatment, as it did in the high concentration ZVI treatment (Figure 4.9). Other daughter products that did not appear in any of the other treatments also appeared in the chromatograms for the medium concentration ZVI microcosms, similar to the high concentration ZVI treatment (Appendix F). This included a compound that eluted at the same time as ethylbenzene. It is unlikely that ethylbenzene was formed as a consequence of ZVI addition; the identity of this compound therefore remains uncertain.

One unusual feature of the medium concentration ZVI results was the unexpectedly abrupt decrease in CF that occurred between days 63 to 133 after the CF had seemingly reached a plateau at approximately 6-10 µmol/bottle. For bottle #2 (Figure 4.18), the decrease occurred between days 63 and day 66, when the CF decreased to below detection. This was before the addition of SDC-9 and B_{12} on day 66 (to all three bottles), so bioaugmentation was not an explanation for the decrease in this bottle. In the other two bottles, however, the decrease occurred after bioaugmentation and addition of B_{12} (between days 163 and 168 in bottle #1 and days 66 and 79 in bottle #3), so this may have facilitated CF removal. Another possible explanation is an analytical artifact; CF eluted very close in time to a considerably larger peak that was presumptively identified as 1,2-DCA. Over time, the difference in retention times between these peaks decreased slightly, such that the CF peak merged into the larger peak and the integrator started reporting the sum of the two peaks, not CF. The large number and magnitude of the

daughter products formed in the medium concentration ZVI bottles made quantification of CF more challenging.

To address the increase in DCM from ZVI facilitated reductive dechlorination of CT and CF, a dose of the DCM enrichment culture was added on day 161. DCM decreased immediately thereafter from 65 µmol/bottle (95 mg/L) to 39 µmol/bottle (57 mg/L) on day 174; this level was comparable to the final concentration of DCM in the high concentration ZVI treatment (Figure 4.9). Additional doses of the DCM culture were added on days 194, 276 and 528, to no avail. It is unclear what inhibited the DCM culture; it was not the DCM concentration, since the culture was acclimated to levels of approximately 500 mg/L.

To address the accumulation of the presumptively identified 1,2-DCA, the microcosms were bioaugmented on day 251 with a 1,2-DCA respiring culture, along with lactate, which the culture uses as its electron donor. However, there was no subsequent decrease in 1,2-DCA. By this time, CT and CF were sufficiently low that they could not have inhibited the activity of the *Dehalococcoides* that are responsible for dihaloelimination of 1,2-DCA to ethene. The lack of activity may indicate that the compound in question was not actually 1,2-DCA, or that some other compound was inhibiting the *Dehalococcoides*.

The average performance of each treatment with respect to CT, CF and DCM is summarized in Figure 4.19. All of the biostimulated and bioaugmented treatments achieved complete or nearly complete removal of CT. The treatment with ZVI added was by far the fastest. The treatment with corn syrup + B_{12} was only marginally slower

than the DHM-1 bioaugmented treatment, while the SDC-9 bioaugmented treatment lagged behind. With respect to CF, the ZVI + SDC-9 bioaugmented treatment was fastest, followed by the corn syrup + B_{12} and DHM-1 bioaugmented treatments. The SDC-9 bioaugmented treatment was slower, but that was likely a consequence of not adding the second dose of B_{12}. No significant removal of CF occurred in the unamended control or the SRB bioaugmented treatment; this may have also been a consequence of not adding a second dose of B_{12}. With respect to DCM, a significant increase occurred in the ZVI treatment that was only partially offset by addition of the DCM enrichment culture. The DCM culture was largely ineffective in the other treatments; it is unclear what was inhibiting the culture.

4.1.3 Low concentration plume (CT ≈ 5 mg/L, CF ≈ 1~2 mg/L)

Results for the AC treatment are shown in Figure 4.20. Percent decreases are summarized in Table 4.1. Over an incubation period of 583 days, decreases in CT and CF averaged 31% and 17%, respectively. DCM and CM remained below 0.050 µmol/bottle throughout the incubation period. CS_2 fluctuated over time (reflecting the lower precision for measuring CS_2 with a flame ionization detector), although the final reading was similar to the initial value. Toluene and ethylbenzene were not present in the low concentration plume.

In the unamended treatment (Figure 4.21), the decreases in CT and CF were not significantly different from the autoclaved controls (Table 4.1; Student's t-test, α=0.05) This is consistent with field observations, i.e., a lack of any appreciable transformation

CT or CF in the low concentration plume. There was also no appreciable change in CS_2.

Biostimulation with corn syrup resulted in complete removal of CT (Figure 4.22). Commensurate with the decrease in CT was an increase in CF from 0.69 µmol/bottle (1.3 mg/L) to 1.59 µmol/bottle (3 mg/L) on day 248. CF levels decreased to 1.04 µmol/bottle (2 mg/L) at the end of the incubation period. The decrease in CT was also accompanied by a noticeable increase in CS_2, which then declined over time to approximately the same level as at the start.

Biostimulation with corn syrup plus B_{12} resulted in complete removal of CT and CF, without pH adjustment of the groundwater (Figure 4.23; Table 4.1). While CT was being consumed, CF increased but then declined to below its MCL by day 522. CS_2 also increased as CT was consumed and subsequently decreased to approximately the same level as at the start of the incubation. The increases in CF and CS_2 were likely a consequence of not adding B_{12} until after the decline in CT started; had the B_{12} been added earlier, it is less likely that there would have been an increase in CF or CS_2.

pH adjustment of the groundwater to circumneutral followed by biostimulation with corn syrup plus B_{12} also resulted in complete removal of CT and CF (Figure 4.24; Table 4.1). The maximum rates of CT and CF removal were faster than for the treatment without pH adjustment. Otherwise, the patterns were similar; i.e., the decrease in CT was accompanied by a rise in CF and CS_2, both of which decreased once the CT was consumed. As with the corn syrup plus B_{12} treatment, B_{12} was not added until after the decrease in CT started, which likely allowed for a transient increase in CF. It should be

noted that corn syrup additions were made later with this treatment than the others; corn syrup was not added until the groundwater pH was stabilized. It is also notable that methane began to accumulate at the end of the incubation period, once CT and CF were below their MCL levels.

The average performance of each treatment with respect to CT, CF and DCM is summarized in Figure 4.25. The three biostimulated treatments achieved complete removal of CT; the treatments with B_{12} added reached the MCL at a faster rate. Only the two treatments with B_{12} added achieved complete biodegradation of CF, with the pH adjusted treatment reaching the MCL several months sooner. None of the treatments achieved complete removal of DCM, although the initial levels were low (0.020-0.030 µmol/bottle or 0.030-0.046 mg/L). DCM ended at close to the initial levels for most of the treatments, with the exception of biostimulation with corn syrup plus B_{12}, in which the level approximately tripled.

4.1.4 Results Summary for Site A

The overall results are summarized in Table 4.2. For the high concentration plume, the only treatment that was effective for CT was addition of ZVI followed by bioaugmentation. CT was completely removed by the ZVI alone. Most of the CF was also removed, but then the activity stalled. DCM levels rose considerably during reaction of the CT and CF with ZVI; an attempt to remove the DCM via bioaugmentation with a DCM-degrading enrichment culture was only partially effective; for reasons that are not yet clear, biodegradation of DCM stalled when the concentration reached 45 mg/L.

Nevertheless, since the plume will migrate into the lower concentration zones downgradient, there would still be ample opportunity to remove the remaining DCM.

For the medium concentration plume, three of the treatments that included addition of B_{12} (M-BB12, M-BIOA and M-BIOB) were effective in completely removing CT and one was effective in completely removing CF (M-BIOA). Bioaugmentation did not improve the rate of CT or CF biodegradation, and therefore, does not appear to be warranted. For DCM, addition of a DCM enrichment culture showed some promise near the end of the incubation period (M-BIOA); additional monitoring would be needed to determine if complete removal is feasible. In the other biostimulation treatments, DCM remained relatively unchanged from its initial concentration. Addition of ZVI was also effective for removing CT and CF in the medium concentration plume. However, this yielded a significant increase in the DCM concentration that was only partially removed by addition of the DCM enrichment culture. Since biostimulation that includes B_{12} avoids this increase (as well as an increase in other products that have not yet been identified), this approach is preferable to ZVI addition. However, cost considerations (primarily for the B_{12}) also need to be taken into account.

For the low concentration plume, biostimulation that included addition of B_{12} was effective in completely removing CT and CF. Increasing the pH of the groundwater improved the rate of removal, although the cost of doing so would need to be weighed against the improvement in rate. The concentration of DCM increased in the treatment without pH adjustment, which is another factor that would need to be taken into account.

No attempt was made to evaluate bioaugmentation of the low concentration plume microcosms to remove the DCM.

4.2 Site B (CT ≈ 20 mg/L, CF ≈ 80 mg/L)

Results for the AC treatment are shown in Figures 4.26; percent decreases are summarized in Table 4.3. Over an incubation period of 716 days, the losses in the autoclaved controls for CF and CT were 21% and 30%, respectively. The initial amount of DCM present was considerably lower and actually increased slightly over time (from 1 µmol/bottle (2 mg/L) to 3 µmol/bottle (4 mg/L)). CS_2 was not detected. The percentage decrease in 1,2-DCA was similar to CT, while the decrease was highest for 1,1,2-TCA (44%). Overall, the magnitude of losses from the AC treatment was considered acceptable, given the long incubation period.

Compared to the AC treatment, the unamended treatment (Figure 4.27) had a similar percent loss for CT (33%; Table 4.3) but significantly higher percent losses for the other compounds (Student's t-test, α=0.05). These results indicate that there was biotic activity in the unamended treatment, although the rate was insufficient to achieve complete removal over the period of time the microcosms were monitored (~2 years).

The vertical rise in CT, CF, and DCM in all of the treatments on day 677 was a consequence of adding 6-8 mL of groundwater (which contained the chlorinated compounds) back to the microcosms. This volume had been removed over time due to repeated sampling to measure sulfate. The reason for closely monitoring sulfate is described below. To restore the ratio of headspace to liquid volume (on which the GC response factors are based), it was necessary to add groundwater back to the microcosms.

The impacts of groundwater withdrawals on the GC response factors of the contaminants for each treatment are summarized in Appendix G; for CT, the impact was less than 1%, while for CF, the maximum impact was 12.3%. In the case of the unamended treatment, the biodegradation rates for CT, CF, and DCM biodegradation did not change appreciably after adding the groundwater.

Biostimulation with corn syrup resulted in nearly complete removal of CT (Figure 4.28; Table 4.3) and a higher percent removal of CF and 1,1,2-TCA compared to the unamended treatment. When groundwater was added back to the microcosms on day 677, the subsequent rate of biodegradation activity was no faster than the previous rate. These results indicate that biostimulation improved the level of biotic activity over the unamended treatment, but may not be sufficient to achieve complete treatment in a timely manner.

Unlike Site A, addition of B_{12} along with corn syrup did not appreciably improve the rate or extent of biodegradation in comparison to addition of only corn syrup (Table 4.3). B_{12} was added twice (Figure 4.29), with no apparent benefit. A potential reason for the lack of response may be the inability to maintain an adequate level of sulfide; the issue of sulfate reduction is discussed below. When groundwater was added back to the microcosms on day 677, the rates of biodegradation were similar to previous activity.

Bioaugmentation with DHM-1 achieved a modest improvement in biodegradation activity (Figure 4.30). CT removal was complete (i.e., below the MCL) and the percent removal of CF was higher than in the other treatments, although it was still not complete

by the end of the incubation period (Table 4.3). It is not clear if the magnitude of improvement would justify the added expense for bioaugmentation.

The lack of improvement in the biodegradation rate for CT and CF from adding B_{12} was unexpected. In numerous previous studies and from the results for Site A, adding B_{12} significantly improved the rate and extent of CT and CF biodegradation and resulted in significantly lower accumulation of chlorinated daughter products. It was hypothesized that a lack of sulfide in the groundwater may be a factor. The activity of DHM-1 and B_{12} is strongly dependent on sulfide being present in the medium (40), with the role of sulfide likely related to providing sufficiently low redox levels to drive continually redox cycling of B_{12} (4). The initial concentration of sulfate in the Site B groundwater was only 0.11 mM. Beginning on day 394, 1.5 mM of sulfate (75 µL of a 1M solution of Na_2SO_4) was added to the bioaugmentation (BIO) treatment; the same dose was provided to the biostimulation (BST) and B_{12} treatments beginning on day 465 (Figure 4.31). Sulfate concentrations were measured after 20-60 days of incubation and when the concentration decreased by at least 0.5 mM, more sulfate was added so that the concentration was returned to approximately 1.5 mM. The bioaugmented treatment consumed the most amount of sulfate (Figure 4.31b); nevertheless, the impact on the rate of CT and CF removal was small. Sulfide was not measured, so it is not known if the dissolved level of sulfide increased as a consequence of sulfate reduction.

Figure 4.32 and Table 4.4 provide summaries of the results for Site B in terms of CT, CF and DCM. The biotic amended treatments achieved CT removal to similar extents and at similar rates. CF was removed in all of the biotic treatments to similar

extents and at similar rates; this result for the unamended microcosms was unexpected, but confirmed the presence of CF biodegrading conditions in situ. For DCM, there was no significant difference in percent removal between the unamended treatment (i.e., CON) and the other live treatments (i.e., BST and BB12). Furthermore, the initial concentration of DCM was considerably lower than for CT and CF.

4.3 Effect of B_{12} to CF Ratio on the Rate of CF Biodegradation by DHM-1

The effect of B_{12} dose on the maximum rate of transformation of 500 mg/L of CF by DHM-1 is shown in Figure 4.33; results for the individual bottles used to generate this figure are provided in Appendix H. Reducing the molar ratio of B_{12} added per mole of CF added from 0.03 mol B_{12} per mol of CF added to 0.01 resulted in a moderate decrease in the maximum CF transformation rate, while the rate fell more quickly below 0.01. Fitting the data to equation 3.1 resulted in a V_{max} of 66±4.6 mg CF/L·d and a B_{12}/K_m ratio of 0.0050±0.0010 mol B_{12} per mol CF (± values indicate 95% confidence intervals). Assuming a yield of 50-60 mg protein/L from the single dose of corn syrup added (40), V_{max} can be normalized to approximately 1.2 mg CF/mg protein·d.

4.4 Effect of pH on the Rate of CF Biodegradation by DHM-1

Maximum CF biodegradation rates for DHM-1 increased with increasing pH from 5.0 to 7.7 (Figure 4.34; results for the individual bottles used to generate this figure are provided in Appendix I). There appeared to be a plateau in the pH range from 6.4 to 7.3, while the rate almost doubled at pH 7.7, reaching 50 mg/L·d. The activity of DHM-1 diminished substantially below pH 6.0 and ceased at pH of 5.0. Lag times (i.e., the time prior to the onset of a maximum rate) decreased as pH increased.

5.0 DISCUSSION

For Site A, in the vicinity of the source zone (i.e., the high concentration plume), bioaugmentation with DHM-1 was the only bioremediation strategy which showed partially effective performance on the biotransformation of CT. However, only one out of three microcosms completed the transformation of CT. No significant degradation of CF happened in any of the treatments for the high concentration plume except the one amended with ZVI. The feasibility of using bioremediation to clean up halogenated solvents in the source zone depends in part on the ability of microbes to grow in the presence of contaminant concentrations that approach their aqueous solubility. It has been proved that DHM-1 has the ability to grow and biodegrade CF at least as high as 500 mg/L (40). Therefore, in this case, it appears that the high concentration of CT is the reason for the lack of activity on CF, via competitive reactions with critical enzymes (49) and/or cellular membrane damage (41). The CT concentration in the source zone plume (~500 mg/L) is well above half of the aqueous solubility of CT and CT is known to inhibit anaerobic bacteria at a concentration as low as 9 mg/L (7). Further work is needed to evaluate the effect of high concentrations of CT on bioaugmentation cultures such as DHM-1.

For the medium concentration plume (CT ~70 mg/L, CF ~24 mg/L), the biostimulation and bioaugmentation treatments with B_{12} added proved to be at least partially effective at removing CT and CF; the bioaugmentation treatment with DHM-1 (M-BIOA) accomplished complete removal (i.e., below the MCL levels). B_{12} was a

crucial factor in enhancing the transformation rate and shifting the pathway away from reductive dechlorination towards hydrolytic and substitutive reactions so that CF and DCM did not accumulate. These observations are consistent with previous studies that demonstrated the benefit of adding catalytic levels of B_{12} to biodegrade CT and CF (3, 7, 23-24, 37). In this study, the benefit of bioaugmenting the medium concentration microcosms yielded only a modest improvement in the outcome, while bioaugmentation (with a culture that preceded DHM-1) was more effective in an experiment conducted by Shan et al. (39). At Site A, the indigenous microbial community has the ability to biodegrade CT and CF when provided with corn syrup and B_{12}. When comparing the treatments that received corn syrup and B_{12} (M-BB12 and M-BIOA) versus lactate (M-BIOB and M-BIOC), it appears that corn syrup was a more effective electron donor for this site. However, since this comparison involved factors in addition to the electron donor (i.e., the culture source), this is not an entirely robust conclusion. From a physiological standpoint, the fermentation of corn syrup is likely to support the growth of a wider range of anaerobes than lactate, based on a greater variety of organic acids produced that would then undergo further acidogenesis (18). In the experiment conducted by Shan et al. (39), corn syrup was also more effective than other tested electron donors including emulsified vegetable oil and lactate. Considering that biodegradation of CT and CF has been found under many different anaerobic conditions and the transformation is mediated by nonspecific cometabolic processes (35), it is possible that most subsurface ecosystems have a latent capacity for CT and CF transformation. Further research is needed to identify the specific conditions needed to

ensure rapid biodegradation of CT and CF degradation without accumulation of lesser chlorinated daughter products.

ZVI was very effective in rapidly transforming CT and CF, primarily to DCM. The accumulation of a lesser chlorinated daughter product is consistent with other studies that have evaluated ZVI treatment of halomethanes (10, 26). In the case of Site A the reaction stopped at DCM (there was no apparent increase in CM or CH_4), which is not a desirable end product. Another shortcoming with ZVI that was evident with Site A was an increase in a substantial number of other volatile reaction products, based on an increase in the number of peaks that eluted during GC analysis of headspace samples. Further work is needed to identify these compounds and ascertain if they pose a toxicological hazard. In the case of the high concentration plume, it is possible that use of ZVI in the source zone would be acceptable, since the daughter products will migrate downgradient and conditions for bioremediation may become more favorable. Nevertheless, identifying the unknown volatile products is needed to fully evaluate the efficacy of ZVI treatment.

In response to the need to treat the large quantity of DCM produced in the ZVI treatments, a DCM enrichment culture capable of biodegrading at least 500 mg/L of DCM was developed in a sediment-free medium. After bioaugmenting the high and medium concentration ZVI treatments, the DCM culture was effective initially but then biodegradation abruptly and unexpectedly stopped. The reason why this phenomenon happened still needs to be identified. Other microbial consortia and strains capable of DCM degradation have only been evaluated under highly controlled and optimum

conditions (13, 30), rather than in the "messy" environment of post-ZVI treatment. The factors that inhibit these DCM degraders still need to be evaluated.

For Site B, the addition of corn syrup accelerated the transformation of CT; however, addition of B_{12} and bioaugmentation failed to accelerate the rate or extent of biodegradation. Compared to the unamended control, biostimulation and bioaugmentation both failed to enhance the degradation of CF. The reason for this unresponsive behavior is thought to be the lack of sulfide, which is needed by DHM-1 for transformation of CF (40). In spite of a high rate of sulfate consumption by the DHM-1 bioaugmented treatment, there was no enhancement of CF biodegradation. If sulfide had been measured, it would have made it possible to determine if sulfide was present or if the sulfide demand of the subsurface material exceeded the amount generated. In this study, 12 mM of sulfate was consumed and presumably reduced to sulfide. Since the microcosms contained 20 g of subsurface material, the sulfide dose was 0.96 mg/g subsurface material. This is well above the dose required to satisfy the sulfide demand for subsurface material at a different hazardous waste site, where direct addition of sulfide was evaluated to reduce hexavalent to trivalent chromium (16). Apparently the sulfide demand for the subsurface material at Site B is even higher.

One of the concerns with using an enrichment culture such as DHM-1 for bioaugmentation is its relatively high requirement for B_{12}. Shan et al. (39-40) used a B_{12} molar dose of 3%, i.e., 0.03 mol B_{12} per mol CF. Varying the B_{12} dose in this study indicated a half saturation value of 0.5%; i.e., the CF biodegradation rate was one-half its maximum at 0.005 mol B_{12}/mol CF. This same B_{12} to CT molar ratio also significantly

improved the rate of CT degradation in a methanogenic sludge consortium, at a CT concentration of 15.4 mg/L (20). Nevertheless, for halomethane concentrations in the hundreds of mg/L, the B_{12} required by DHM-1 is several orders of magnitude higher than what is used to grow *Dehalococcoides* (25). Further work is needed to find a lower cost alternative to highly purified B_{12}.

Aquifer pH is a significant concern for bioaugmentation, since many cultures lose their effectiveness at pH levels below 6 or above 8. The highest pH evaluated in this study was 7.6, which yielded a significantly higher rate than in the circumneutral pH region (i.e., 6.3-7.3; Figure 4.34). CF biodegradation rates decreased significantly below 6.0 and activity essentially ceased at pH 5.0. This is similar to the behavior of many *Dehalococcoides* enrichment cultures used for bioaugmentation of chlorinated ethene plumes. The difficulties associated with adjusting aquifer pH include non-homogenous distribution of the buffering agent and the potential for clogging due to precipitation when pH is increased. The need to control aquifer pH is a factor that must be considered when evaluating the use of bioaugmentation with a culture such as DHM-1.

6.0 CONCLUSIONS

Based on the results of this study, the following conclusions were reached for bioremediation of Site A:

1. Addition of ZVI followed by bioaugmentation was the most promising approach evaluated for removal of CT, and CF from the high concentration plume. This strategy achieved complete removal of CT, nearly complete removal of CF and partial removal of the DCM that accumulated during reaction of the CT and CF with ZVI. There is still a need to determine if the other volatile compounds formed during reaction with ZVI pose a concern for downgradient remediation.
2. Biostimulation with corn syrup plus B_{12} was effective for removal of CT and almost all of the CF from the medium concentration plume. Bioaugmentation did not improve the rate of transformation; however, addition of DHM-1 ensured complete removal of CF. SDC-9 and a SRB enrichment culture developed for use in this study were less effective for removal of CT and CF in the medium concentration microcosms.
3. An anaerobic enrichment culture that grows on DCM as its sole carbon and energy source was successfully developed. It was acclimated to consume up to 500 mg/L of DCM. Use of the culture for bioaugmentation to remove DCM was partially effective; additional work is needed to optimize the use of the culture. Although ZVI plus bioaugmentation removed CT and CF at a faster rate, the

accumulation of DCM was a concern that was only partially addressed by bioaugmentation with the DCM enrichment culture.

4. For the low concentration plume, biostimulation with corn syrup plus B_{12} was the most promising approach evaluated for removal of CT and CF. Adjusting the pH of the groundwater improved the rate of transformation, although this must be weighed against the cost of in situ pH adjustment. No attempt was made to remove the low level of DCM present in the low concentration plume.

Based on the results of this study, the following conclusions were reached for bioremediation of Site B:

1. Biostimulation with corn syrup was effective in removing CT; however, addition of B_{12} and bioaugmentation did not appreciably enhance the rate or extent of CT biodegradation.
2. Biostimulation and bioaugmentation did not improve the rate of CF removal over the unamended treatment. The rate of CF removal was slow and consistent in the biotic treatments.
3. None of the amendments were effective in removing DCM, although it was present at a lower concentration than CT and CF.
4. Attempts to stimulate the effectiveness of B_{12} and bioaugmentation by generating sulfide via sulfate reduction were not effective. Although 12 mM of sulfate was consumed in the treatment bioaugmented with DHM-1, there was no enhancement in the rate or extent of CT or CF biodegradation. This suggests that

the subsurface material has a considerable sulfide demand that needs to be satisfied before the effectiveness of B_{12} and DHM-1 can be realized.

Based on the results of this study, the following conclusions were reached with respect to further characterization of the DHM-1 bioaugmentation culture:

1. Maximum CF biodegradation rates for DHM-1 increased with increasing pH from 5.0 to 7.7. Its activity is severely inhibited by pH levels below 6.0. At pH 5.0, it lost its ability to biotransform CF. Between pH 6.4 and 7.3, the rate of CF biodegradation by DHM-1 is somewhat stable; however, it increases rapidly between pH 7.3 and 7.7. Given the inhibitory effect of pH levels below 6.0, the pH of the groundwater should be adjusted within the neutral range for successful bioaugmentation to occur. The sensitivity of DHM-1 to low pH values must be considered for application in aquifers that have pH levels below 6.
2. Maximum CF biodegradation rates for DHM-1 increased with increasing vitamin B_{12} dose. The relationship between the B_{12} to CF ratio and the rate of CF biodegradation can be described by a modification of the Michaelis-Menten kinetics model. A V_{max} of 66±4.6 mg CF/L·d and a B_{12}/K_m ratio of 0.0050±0.0010 mol B_{12} per mol CF were obtained from fitting experimental results to this model. This information can help in the selection of a cost-effective dose for B_{12} when it is used as an amendment to facilitate bioremediation of chlorinated methanes.

TABLES

Table 3.1 Media used for enrichment cultures[a].

	Type of Media		
Compound	**DHM-1 Culture**	**SRB Culture**	**DCM Culture**
K_2HPO_4	410	410	525
$NaHCO_3$	4000	4000	800
NH_4Cl	300	300	535
$MgCl_2 \cdot 6H_2O$	400	400	-
$MgSO_4 \cdot 7H_2O$	-	-	125
$CaCl_2 \cdot 2H_2O$	150	150	47
$ZnSO_4 \cdot 7H_2O$	-	-	0.42
$MnCl_2 \cdot 4H_2O$	0.1	0.1	2
$CoCl_2 \cdot 6H_2O$	0.19	0.19	3
$Na_2MoO_4 \cdot 2H_2O$	0.036	0.036	-
KCl	300	300	-
$Na_2S \cdot 9H_2O$	120	120	240
$FeCl_2 \cdot H_2O$	-	-	162.6
$FeCl_2 \cdot 4H_2O$	1.5	1.5	-
$ZnCl_2$	0.07	0.07	-
H_3BO_4	0.006	0.006	0.6
$NiCl_2 \cdot 6H_2O$	0.024	0.024	1.5
$CuCl_2 \cdot 2H_2O$	0.002	0.002	0.2
Na_2SeO_3	-	-	0.04
$Na_2SeO_3 \cdot 5H_2O$	0.005	0.005	-
$Al_2(SO_4)_3 \cdot 16H_2O$	-	-	0.2
Resazurin	1	1	1
Yeast Extract	50	50	50
Na_2SO_4	-	5268.2	-

[a] All values in mg/L.

Table 3.2 Experimental design for Site A [a].

Plume Conc.	Treatment ID	Treatment Description	SM Type	GW Type	CT	CS	Lac	B_{12}	Bioaugmentation Culture A	B	C	D	E	ZVI
High	H-AC	Autoclaved control	PZ-95	PZ-95-2	yes	-	-	-	-	-	-	-	-	-
High	H-WC	Water control	-	-	yes	-	-	-	-	-	-	-	-	-
High	H-Con	Unamended control	PZ-95	PZ-95-2	yes	-	-	-	-	-	-	-	-	-
High	H-BST	Biostimulation	PZ-95	PZ-95-2	yes	yes	-	-	-	-	-	-	-	-
High	H-BB12	Biostimulation + B_{12}	PZ-95	PZ-95-2	yes	yes	-	yes	-	-	-	-	-	-
High	H-BIOA	Bioaugmentation A	PZ-95	PZ-95-2	yes	yes	-	yes	yes	-	-	-	-	-
High	H-BIOB	Bioaugmentation B	PZ-95	PZ-95-2	yes	-	yes	yes	-	yes	-	-	-	-
High	H-BIOC	Bioaugmentation C	PZ-95	PZ-95-2	yes	-	yes	yes	-	-	yes	-	-	-
High	H-ZBIO	ZVI + bioaugmentation	PZ-95	PZ-95-2	yes	yes	yes	yes	yes	-	-	yes	yes	yes
Medium	M-AC	Autoclaved control	TP-1	TP-4	yes	-	-	-	-	-	-	-	-	-
Medium	M-Con	Unamended control	TP-1	TP-4	yes	-	-	-	-	-	-	-	-	-
Medium	M-BST	Biostimulation	TP-1	TP-4	yes	yes	-	-	-	-	-	-	-	-
Medium	M-BB12	Biostimulation + B_{12}	TP-1	TP-4	yes	yes	-	yes	-	-	-	-	-	-
Medium	M-BIOA	Bioaugmentation A	TP-1	TP-4	yes	yes	-	yes	yes	-	-	-	yes	-
Medium	M-BIOB	Bioaugmentation B	TP-1	TP-4	yes	-	yes	yes	-	yes	-	-	yes	-
Medium	M-BIOC	Bioaugmentation C	TP-1	TP-4	yes	-	yes	yes	-	-	yes	-	-	-
Medium	M-ZBIO	ZVI + bioaugmentation	TP-1	TP-4	yes	-	yes	yes	-	yes	-	yes	yes	yes
Low	L-AC	Autoclaved control	PZ-12	PZ-12	yes	-	-	-	-	-	-	-	-	-
Low	L-Con	Unamended control	PZ-12	PZ-12	yes	-	-	-	-	-	-	-	-	-
Low	L-BST	Biostimulation	PZ-12	PZ-12	yes	yes	-	-	-	-	-	-	-	-
Low	L-BB12	Biostimulation + B_{12}	PZ-12	PZ-12	yes	yes	-	yes	-	-	-	-	-	-
Low	L-ABST	Biostim + B_{12} + pH	PZ-12	PZ-12	yes	yes	-	yes	-	-	-	-	-	-

[a] SM = subsurface material; GW = groundwater; CS = corn syrup; Bioaugmentation culture A = DHM-1; B= SDC-9; C = SRB enrichment culture; D = 1,2-DCA enrichment culture; E = DCM enrichment culture. The quantities added to each bottle are specified in the text.

Table 3.3 Summary of the CT and CF concentrations for Site A (mg/L).

Contaminant	High	Medium	Low
CT	400	50	5
CF	340	20	1

Table 3.4 Experimental design for Site B [a].

Treatment ID	Treatment Description	CS	B_{12}	DHM-1
AC	Autoclaved control	-	-	-
Con	Unamended control	-	-	-
BST	Biostimulation	yes	-	-
BB12	Biostimulation + B_{12}	yes	yes	-
BIO	Bioaugmentation	yes	yes	yes

[a] CS = corn syrup.

Table 4.1 Summary of percent removals of CT, CF, DCM, CS_2, toluene and ethylbenzene[a] for Site A.

Treatment	Incubation Time Time (d)	CT	CF	DCM	CS_2	Toluene	Ethylbenzene
H-AC	577	28.9±2.2	28.2±2.8	25.9±4.5	23.5±2.1	52.0±2.7	59.3±3.5
H-WC	412	28.1±0.9	35.8±2.8	20.9±2.6	-	-	-
H-Con	577	25.2±3.2	28.7±1.6	24.0±1.8	25.2±0.9	50.6±1.2	60.2±3.2
H-BST	577	24.0±6.7	27.8±2.3	21.7±1.4	16.7±7.7	53.6±2.9	64.1±1.9
H-BB12	576	32.4±10.3	30.4±3.8	27.4±1.5	26.8±4.9	53.5±3.3	56.0±12.3
H-BIOA	570	51.6±42.0	31.9±2.2	28.2±2.1	34.9±10.9	55.0±5.4	62.4±6.3
H-BIOB	576	24.4±2.8	28.0±1.5	23.2±1.3	22.3±1.9	44.0±12.2	57.4±2.7
H-BIOC	570	20.7±0.9	28.3±1.4	24.9±1.2	23.1±1.1	47.7±1.4	57.0±5.8
H-ZBIO	570	100.0±0.0	96.6±0.7	+[b]	42.1±14.7	43.8±22.1	68.6±4.9
M-AC	609	22.7±1.6	14.2±1.6	15.2±1.9	20.9±1.1	43.2±3.5	-
M-Con	609	32.5±8.0	19.5±4.2	33.3±13.0	32.4±17.1	44.6±3.5	-
M-BST	609	95.8±4.9	+	27.0±2.4	26.5±1.1	42.8±0.4	-
M-BB12	609	100.0±0.0	98.1±3.3	21.0±1.6	24.3±2.8	46.6±3.7	-
M-BIOA	599	100.0±0.0	100.0±0.0	37.3±3.9	19.8±1.4	46.2±6.5	-
M-BIOB	599	100.0±0.0	88.1±20.6	27.8±10.3	20.8±2.1	46.4±7.4	-
M-BIOC	599	94.2±10.0	6.0±14.4	17.1±0.7	18.4±1.6	43.9±1.9	-
M-ZBIO	609	100.0±0.0	100.0±0.0	+	66.7±4.2	-	-
L-AC	583	31.4±3.3	17.0±14.6	+	+	-	-
L-Con	583	32.4±2.3	26.5±9.6	63.7±28.9	41.1±13.2	-	-
L-BST	583	100.0±0.0	+	+	+	-	-
L-BB12	583	100.0±0.0	100.0±0.0	+	+	-	-
L-ABST	583	100.0±0.0	100.0±0.0	+	11.2±17.0	-	-

[a] Average of triplicates ± one standard deviation. [b] + indicates an increase occurred relative to the initial concentration.

Table 4.2 Summary of treatment effectiveness of removing CT, CF and DCM[a] for Site A.

Plume	Treatment	CT	CF	DCM
High Concentration	H-Con	not effective	not effective	not effective
	H-BST	not effective	not effective	not effective
	H-BB12	not effective	not effective	not effective
	H-BIOA	partially effective	not effective	not effective
	H-BIOB	not effective	not effective	not effective
	H-BIOC	not effective	not effective	not effective
	H-ZBIO	effective	partially effective	partially effective
Medium Concentration	M-Con	not effective	not effective	not effective
	M-BST	partially effective	not effective	not effective
	M-BB12	effective	partially effective	not effective
	M-BIOA	effective	effective	not effective
	M-BIOB	effective	partially effective	not effective
	M-BIOC	partially effective	not effective	not effective
	M-ZBIO	effective	effective	partially effective
Low Concentration	L-Con	not effective	not effective	partially effective
	L-BST	effective	not effective	not effective
	L-BB12	effective	effective	not effective
	L-ABST	effective	effective	not effective

[a] Effective = removal to below the MCL; partially effective = >50% decrease in the maximum concentration; not effective = <50% decrease in the maximum concentration.

Table 4.3 Summary of percent removals of CT, CF, DCM, 1,2-DCA, and 1,1,2-TCA[a] for Site B.

Treatment	Incubation Time	CT	CF	DCM	1,2-DCA	1,1,2-TCA
AC	716	29.6±7.1	20.8±1.4	+	28.5±3.1	43.7±9.6
CON	757	33.2±19.3	57.5±12.2	40.2±12.4	67.2±4.5	70.5±4.6
BST	757	97.7±2.9	71.6±3.1	26.8±15.8	76.4±2.4	80.8±2.7
BB12	757	98.8±2.1	66.5±7.7	22.4±12.1	71.8±3.5	74.0±4.0
BIO	757	100.0±0.0	76.7±2.8	57.1±9.6	77.6±4.1	82.3±3.6

[a] Average of triplicates ± one standard deviation. [b] + indicates an increase occurred relative to the initial concentration.

Table 4.4 Summary of treatment effectiveness of removing CT, CF and DCM[a] for Site B .

Treatment	CT	CF	DCM
Con	not effective	partially effective	not effective
BST	partially effective	partially effective	not effective
BB12	partially effective	partially effective	not effective
BIO	effective	partially effective	partially effective

[a] Effective = removal to below the MCL; partially effective = >50% decrease in the maximum concentration; not effective = <50% decrease in the maximum concentration.

FIGURES

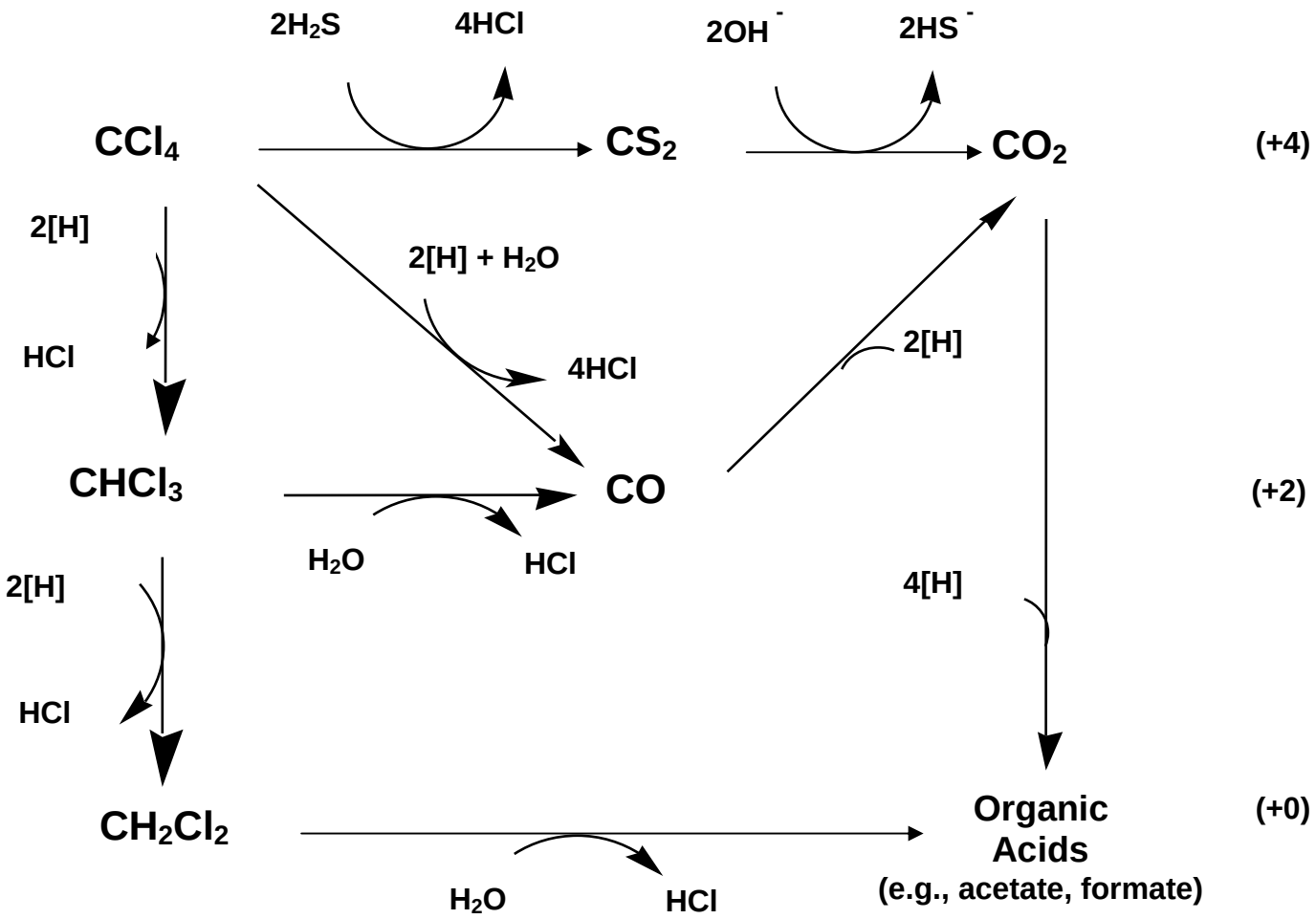

Figure 1.1 Pathways for biotransformation of CT and CF under low redox conditions.

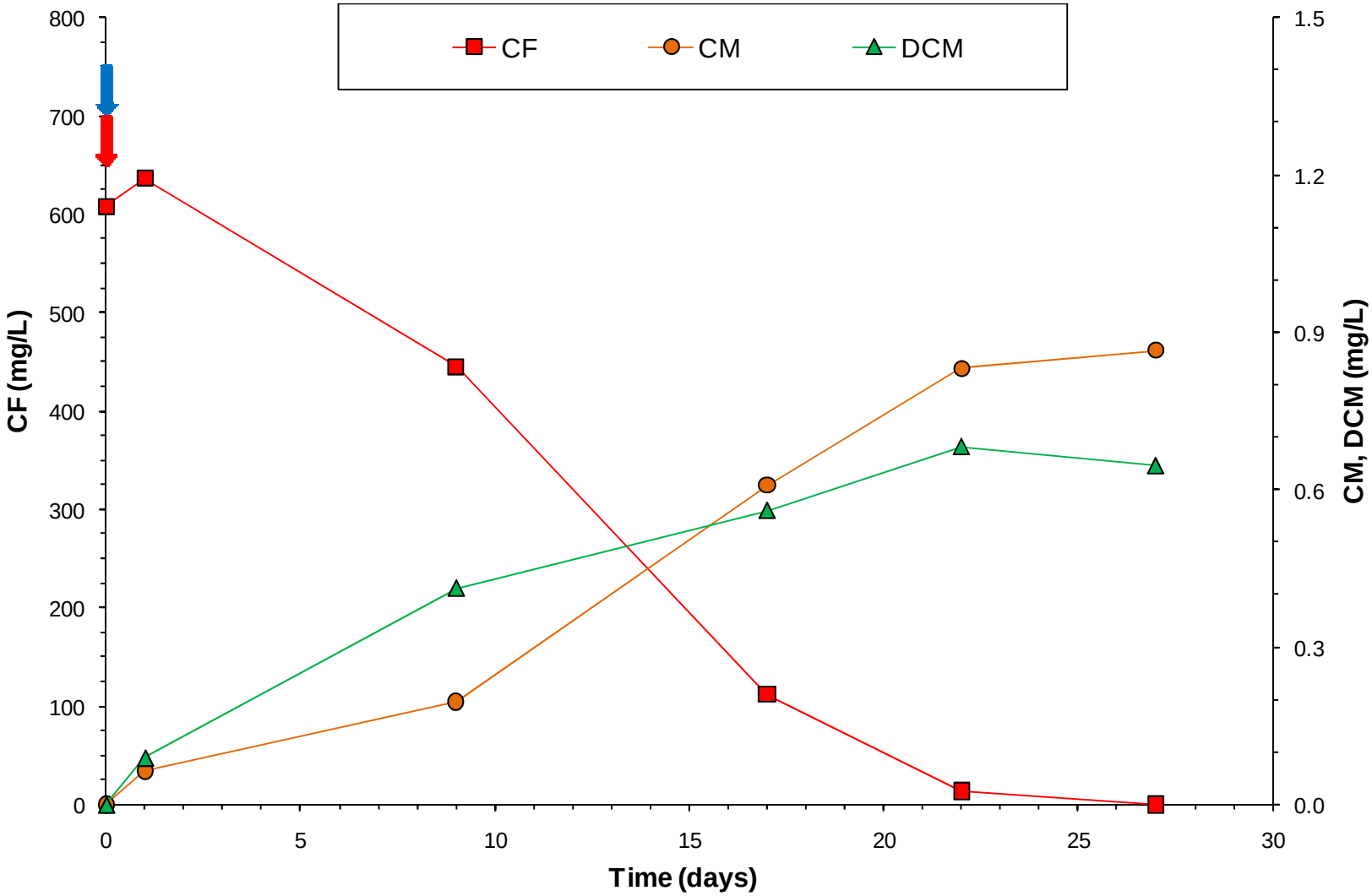

Figure 3.1 Behavior of the DHM-1 culture; ↓ = corn syrup addition, ↓ = B_{12} addition.

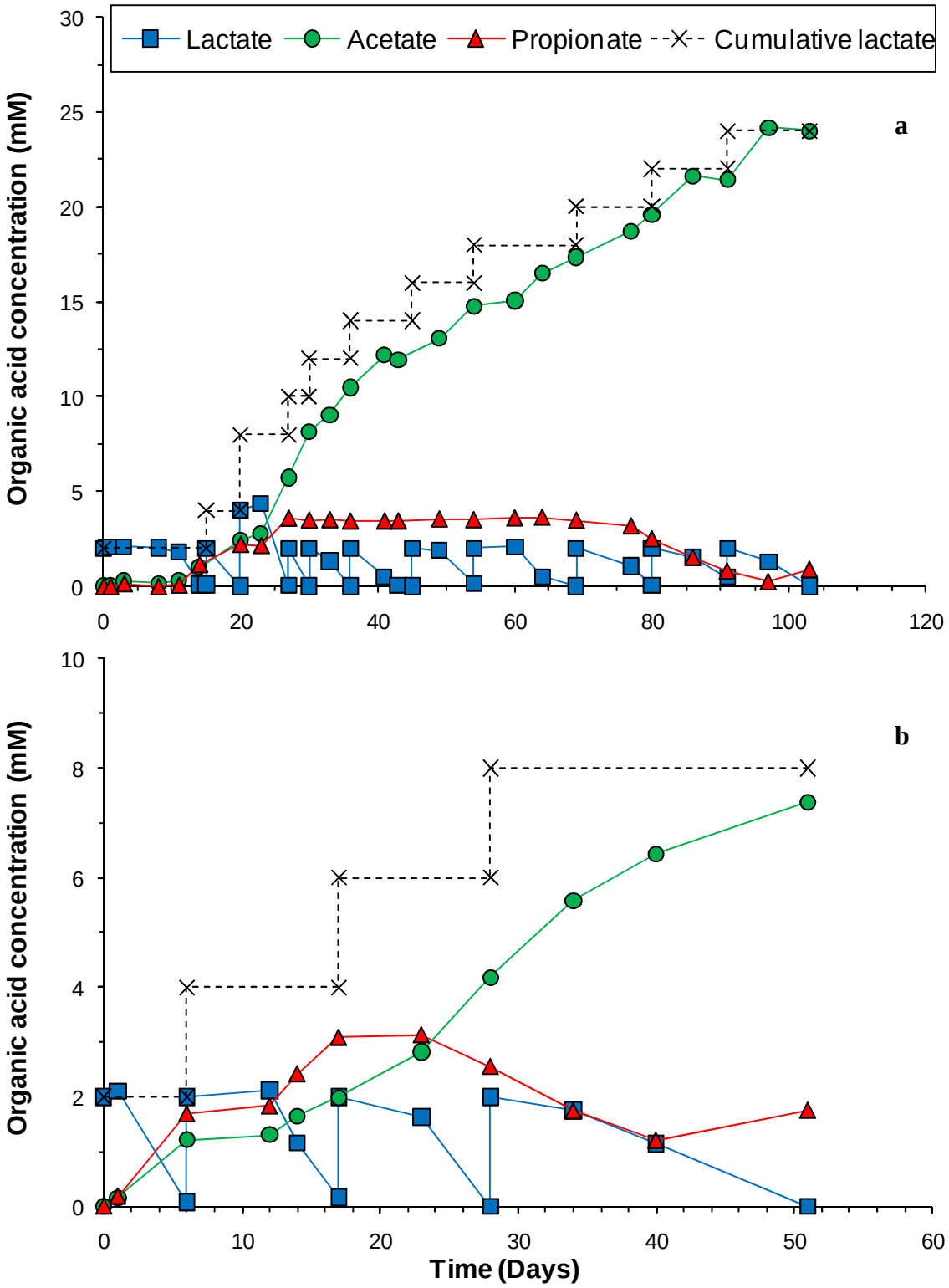

Figure 3.2 Behavior of the SRB culture (a) for the enrichment bottle, and (b) for the sediment-free culture.

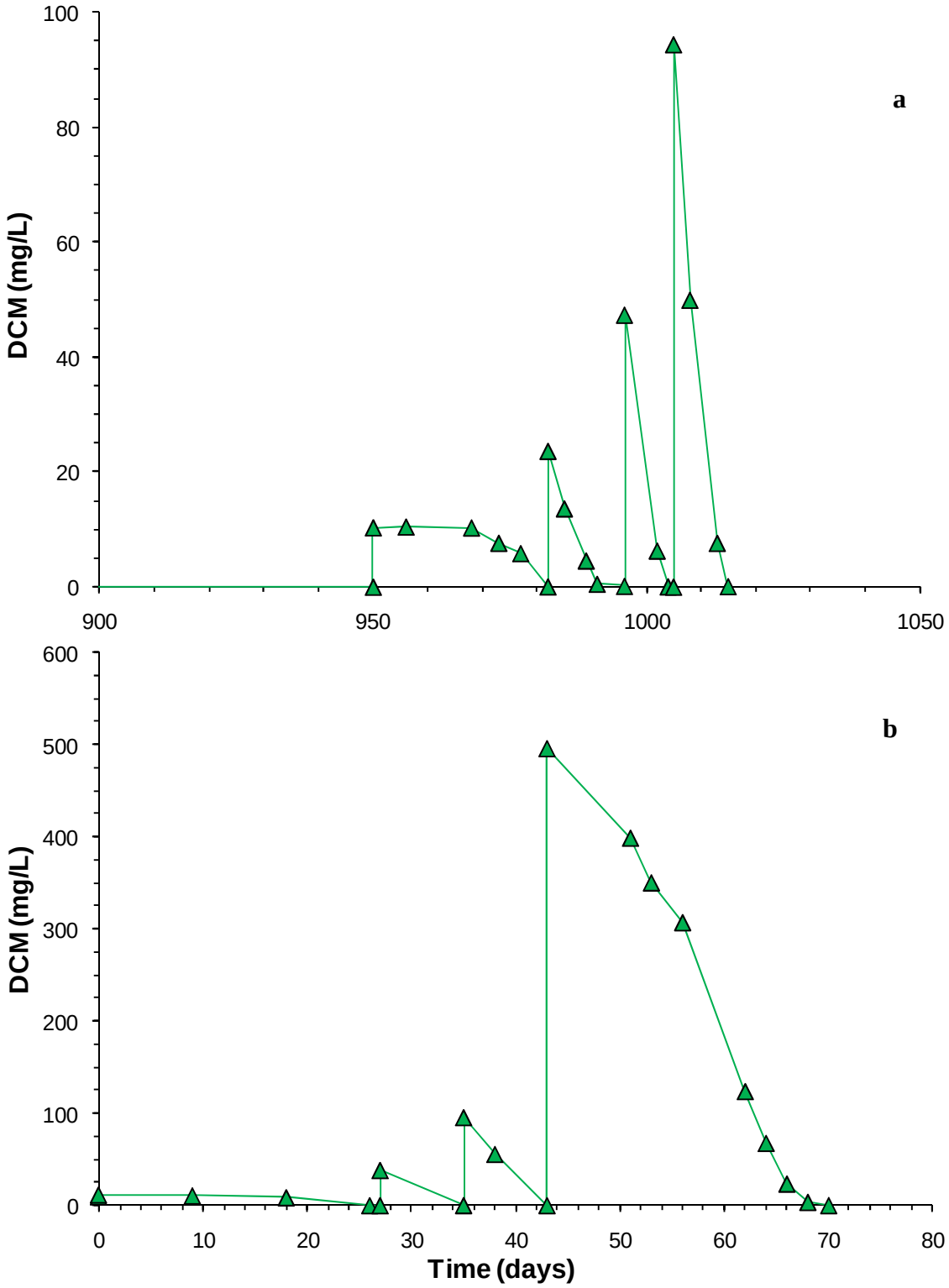

Figure 3.3 Behavior of the DCM culture (a) for the enrichment bottle, and (b) for the sediment-free culture.

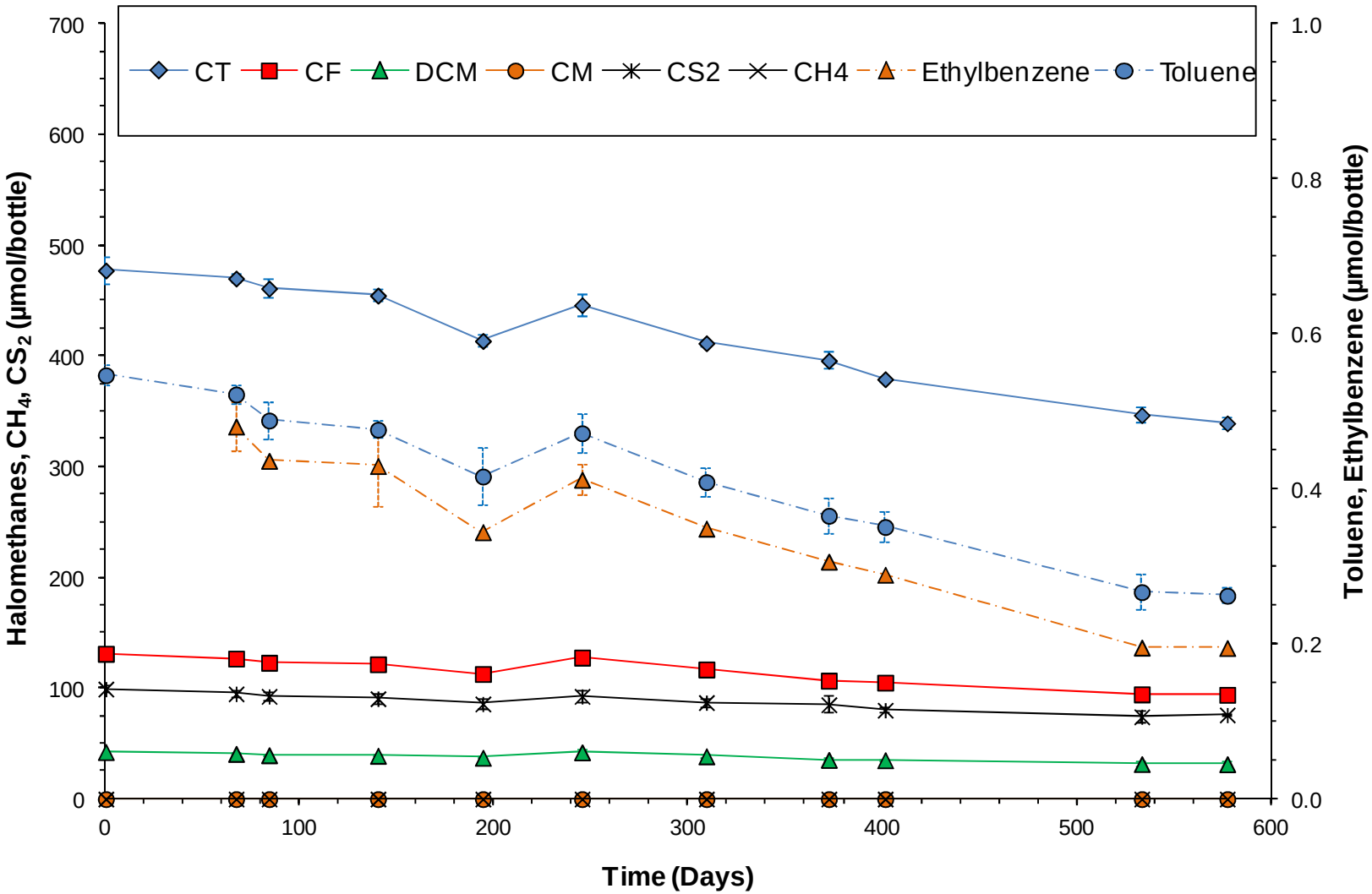

Figure 4.1 Results for Site A, high concentration plume, autoclaved control treatment (average of triplicate microcosms; error bars indicate one standard deviation).

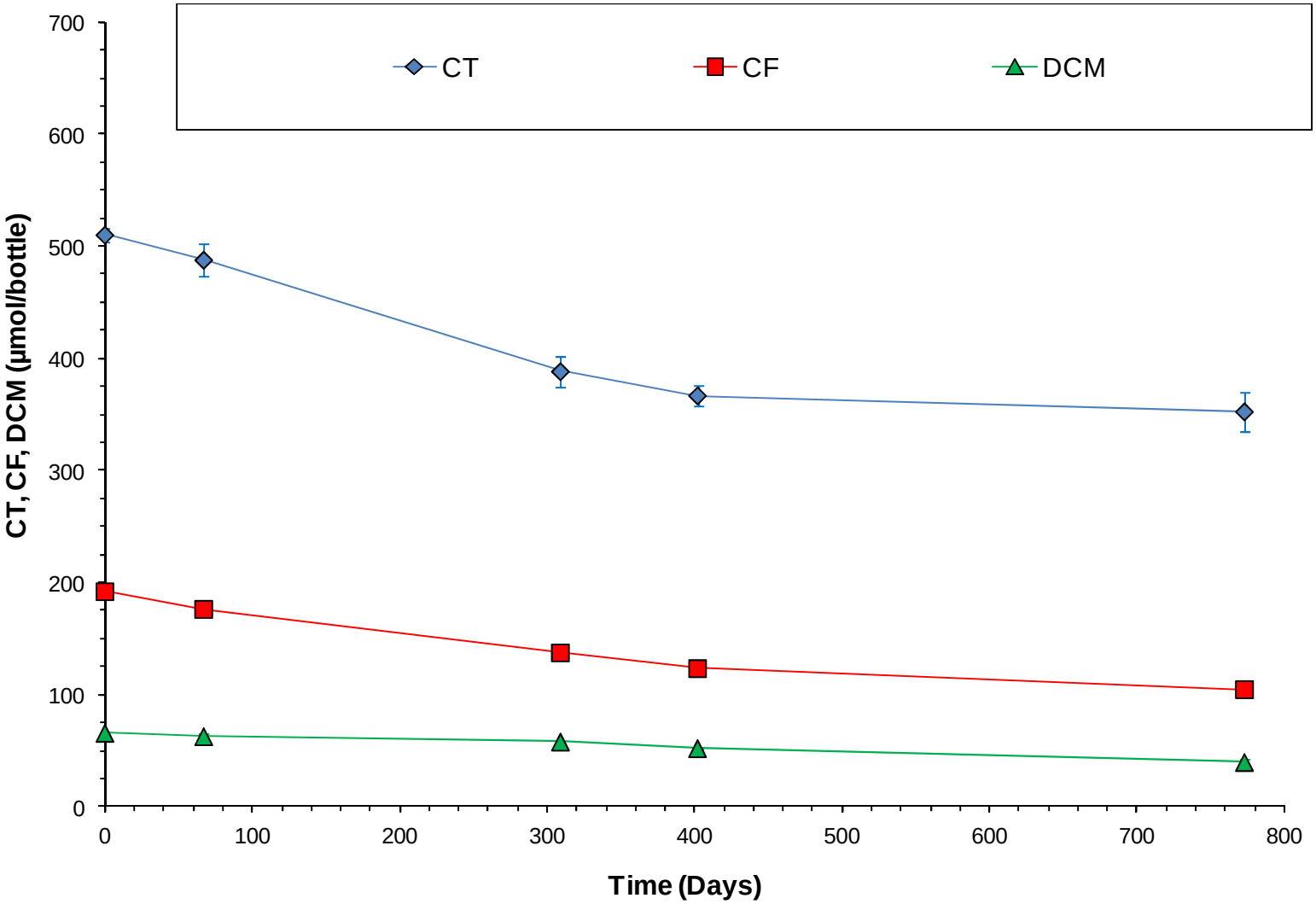

Figure 4.2 Results for Site A, high concentration plume, water control treatment (average of triplicate microcosms; error bars indicate one standard deviation).

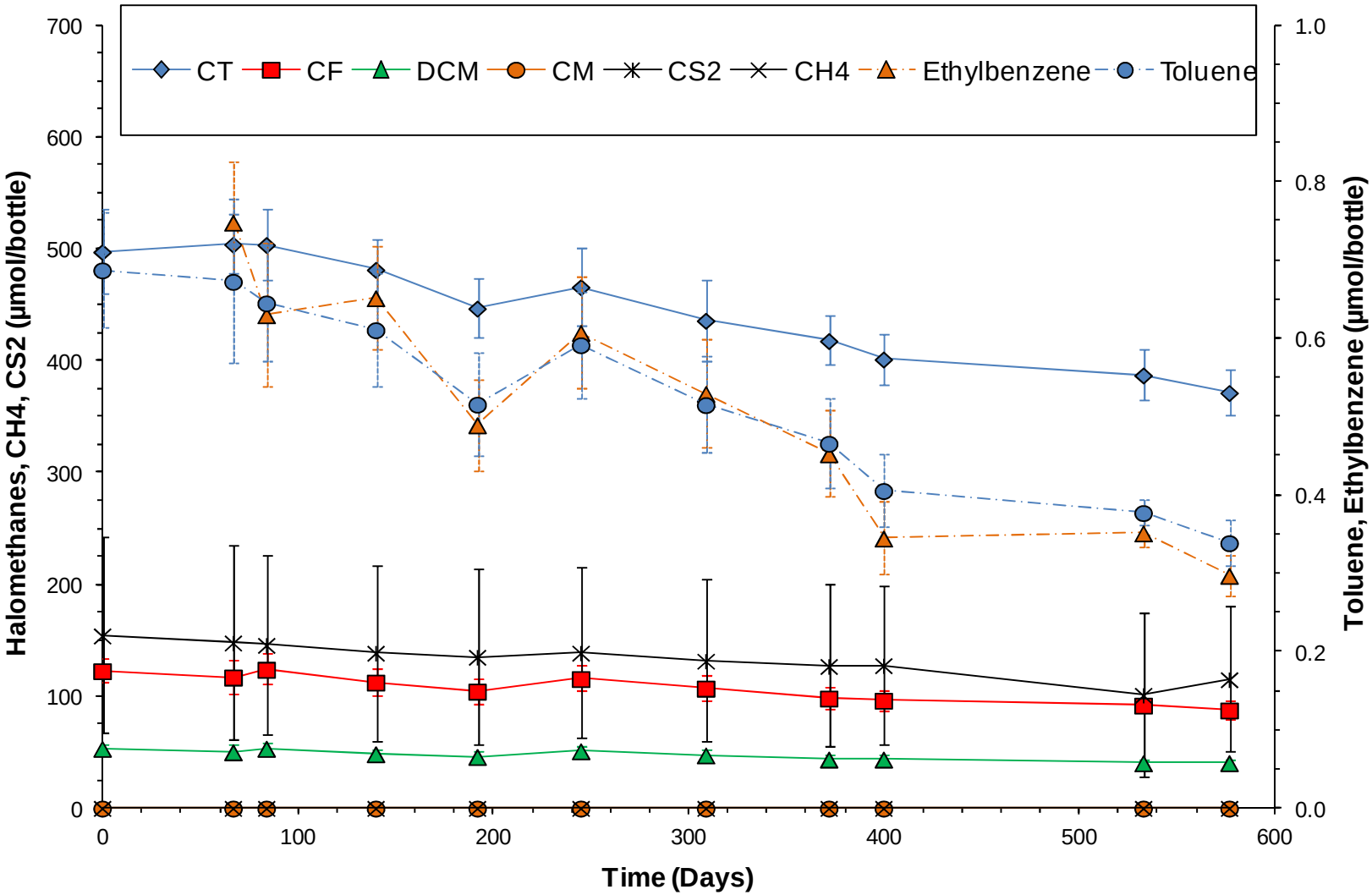

Figure 4.3 Results for Site A, high concentration plume, unamended treatment (average of triplicate microcosms; error bars indicate one standard deviation).

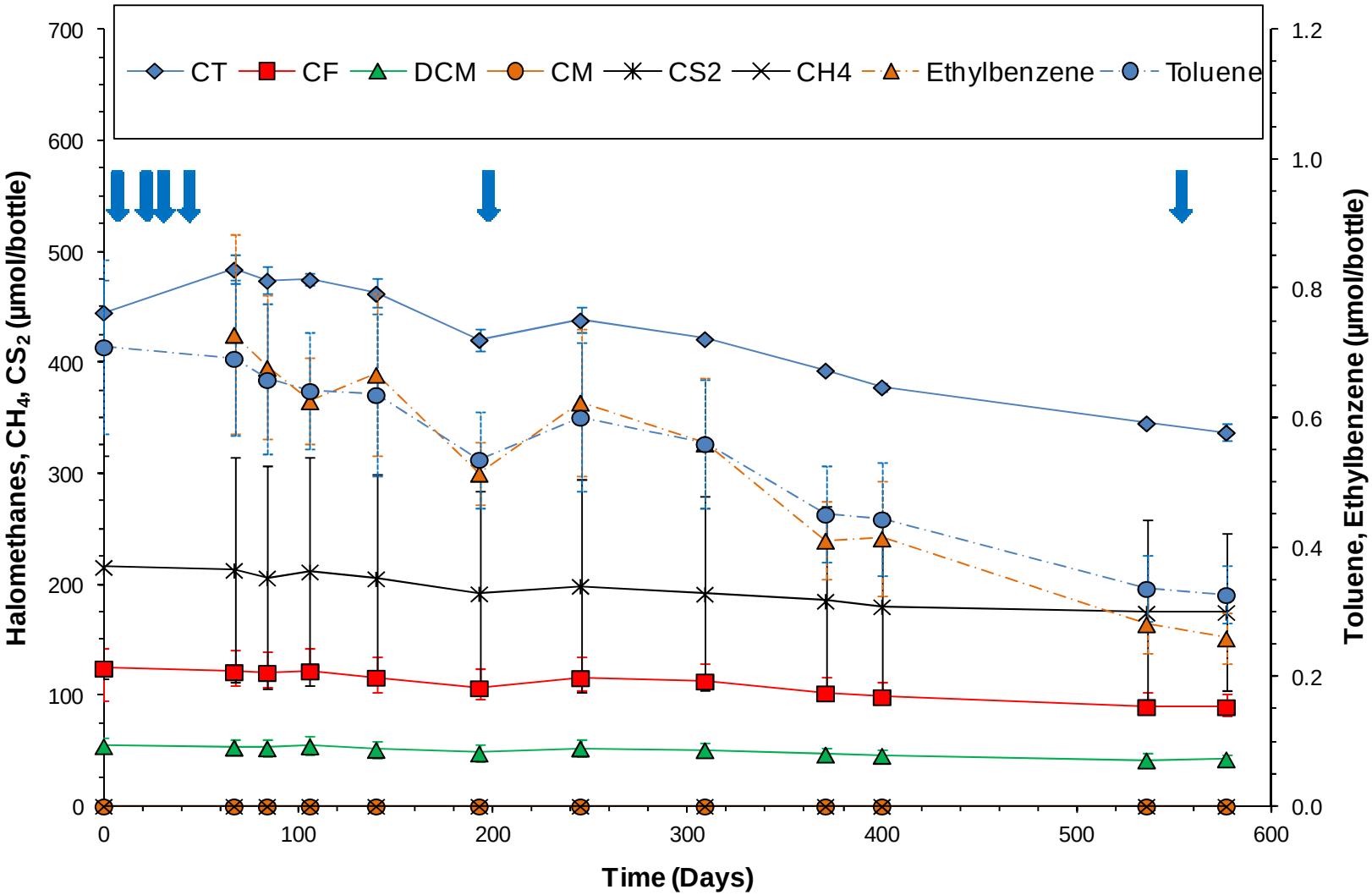

Figure 4.4 Results for Site A, high concentration plume, biostimulation with corn syrup (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of corn syrup.

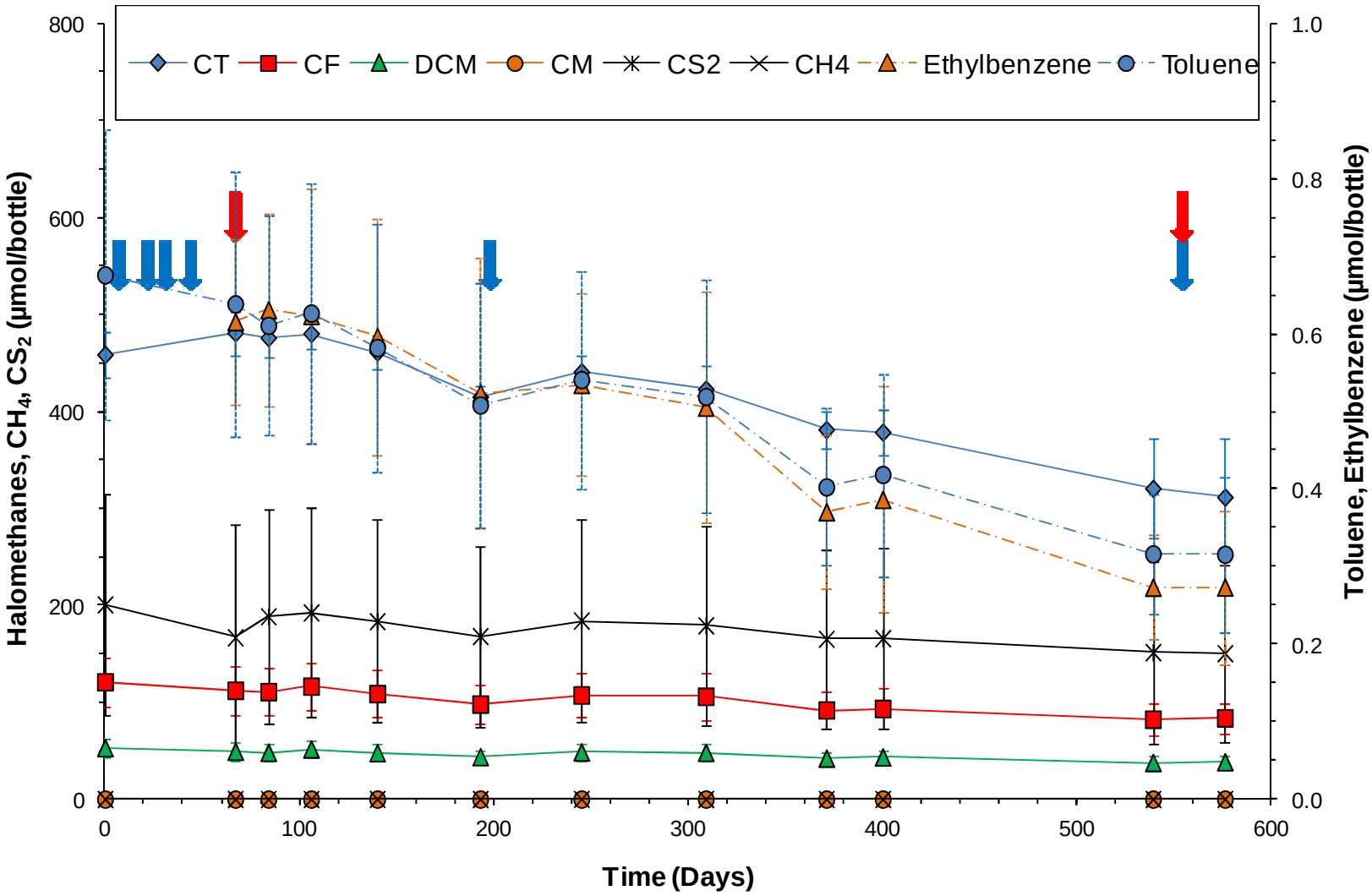

Figure 4.5 Results for Site A, high concentration plume, biostimulation with corn syrup + B_{12} (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of corn syrup; ⬇ = addition of B_{12}.

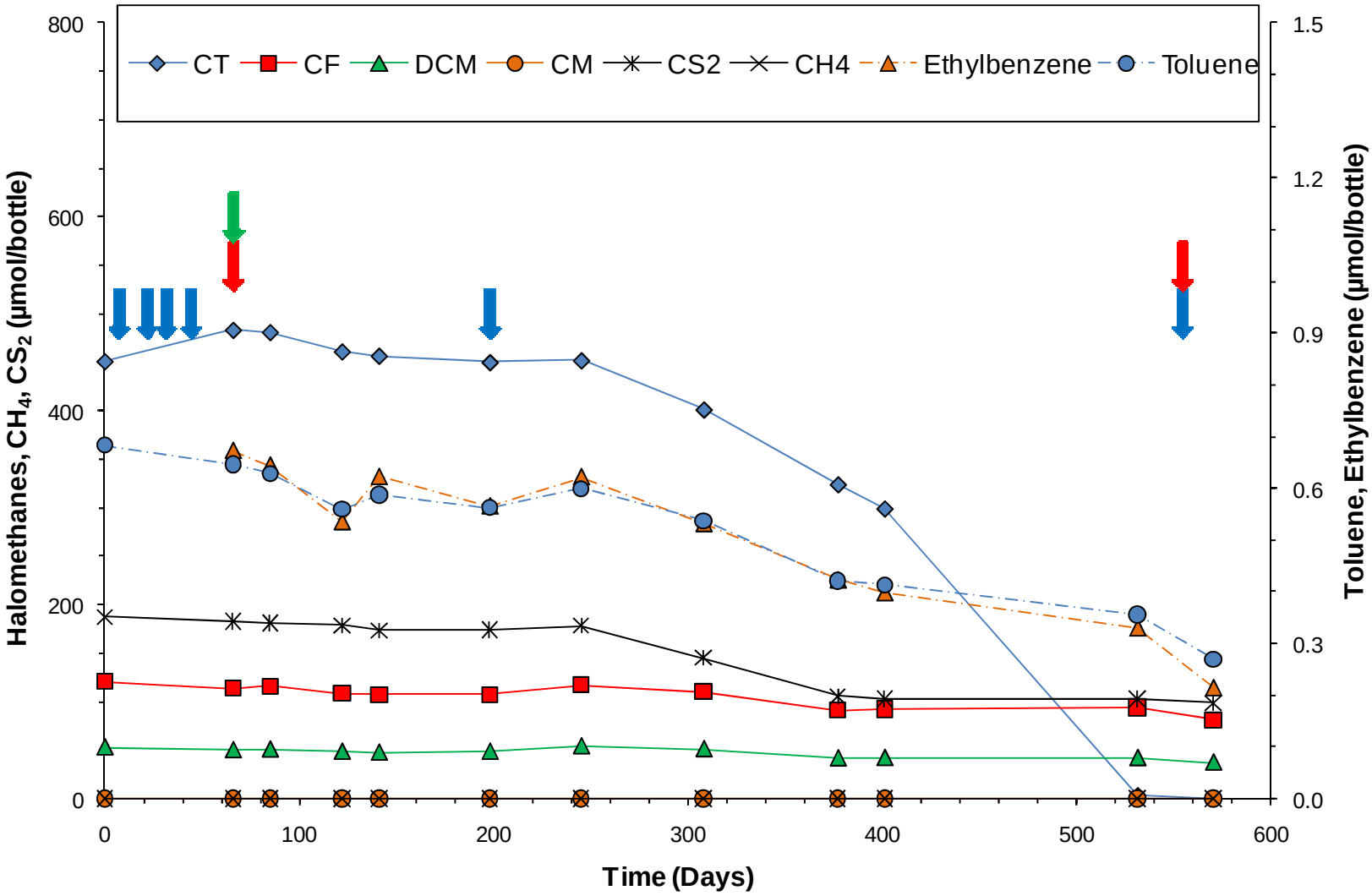

Figure 4.6 Representative results for Site A, high concentration plume, bioaugmentation treatment A (bottle #3); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of DHM-1.

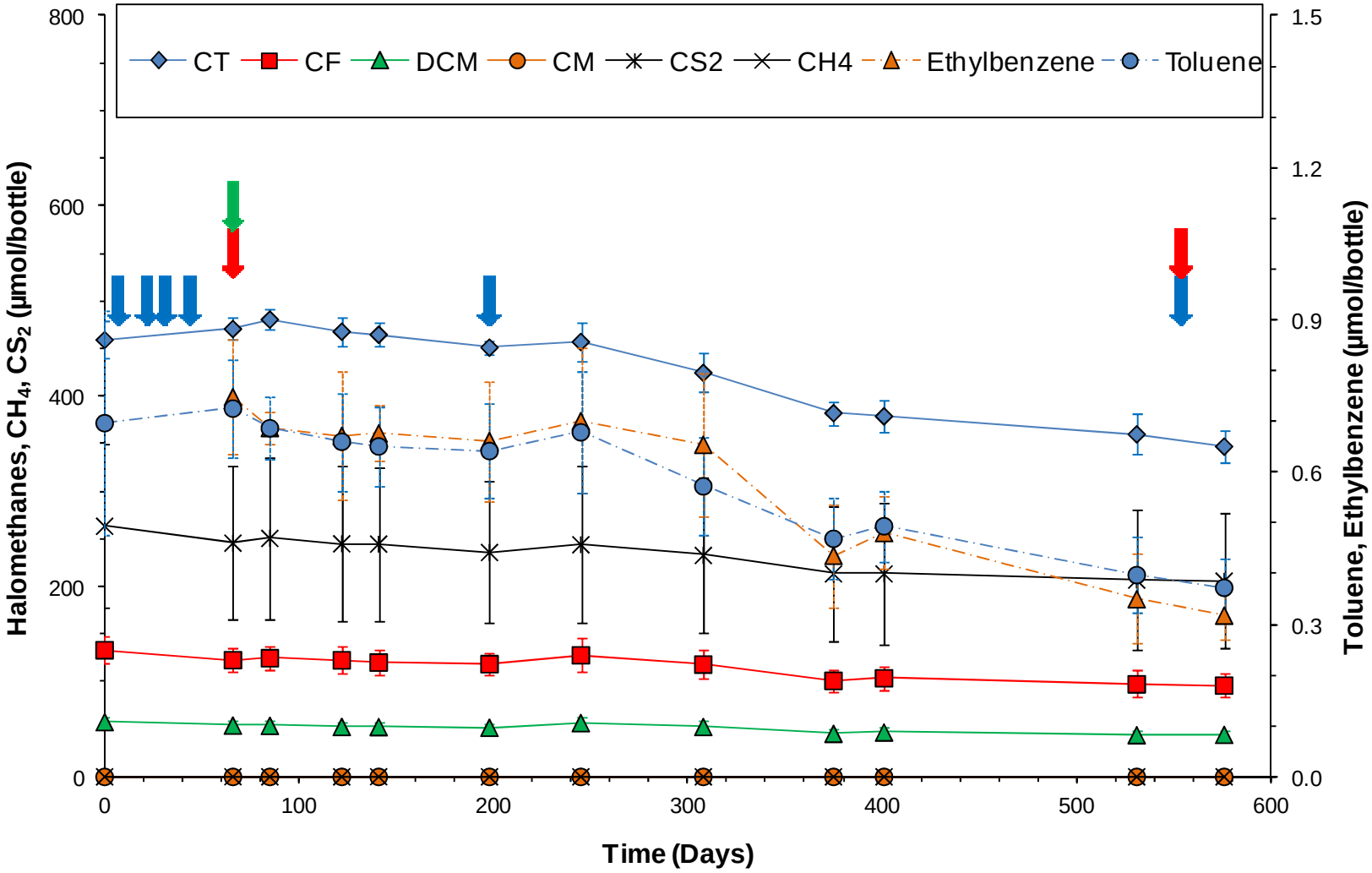

Figure 4.7 Representative results for Site A, high concentration plume, bioaugmentation treatment B (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of lactate; ⬇ = addition of B_{12}; ⬇ = addition of SDC-9.

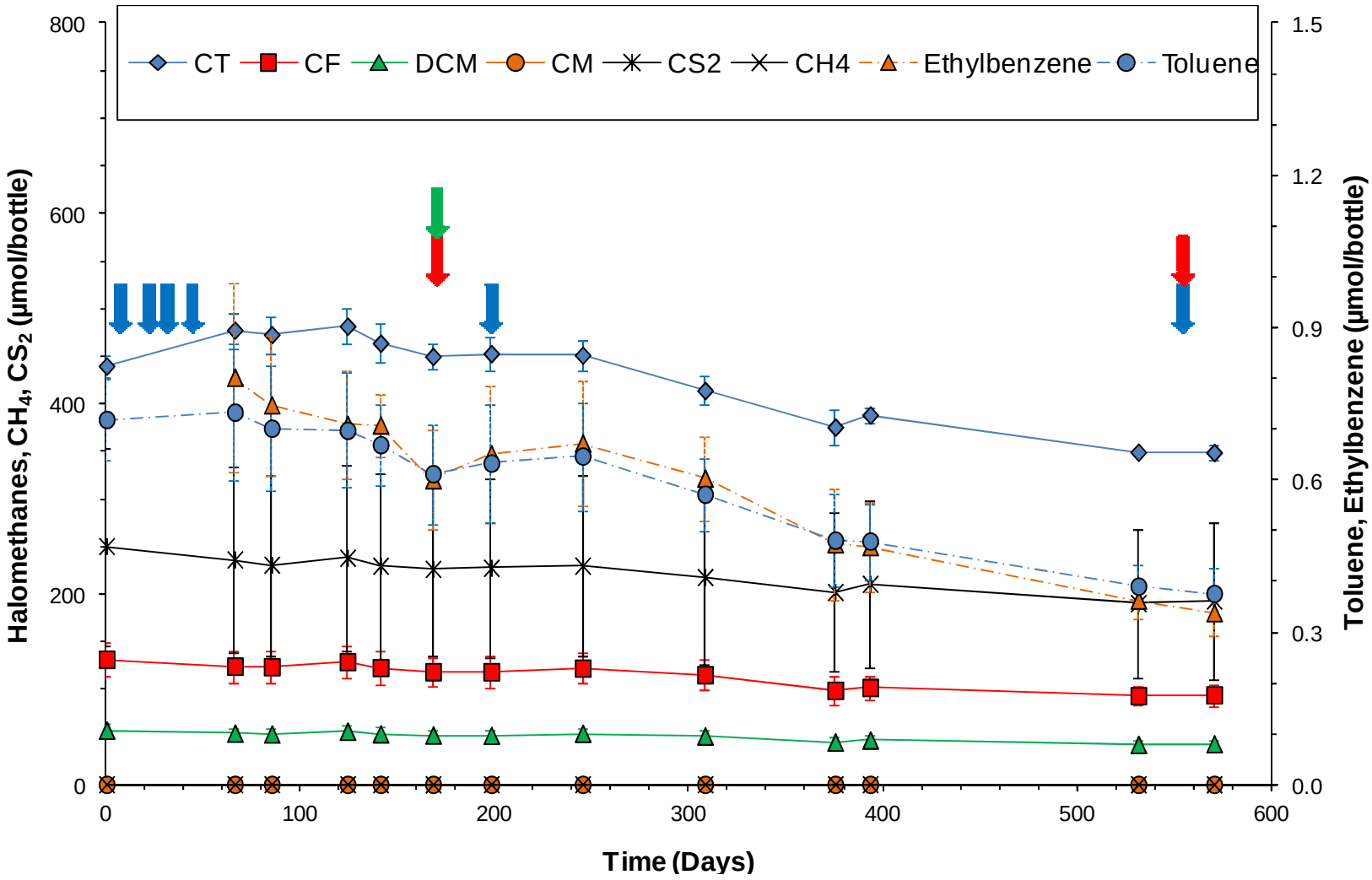

Figure 4.8 Representative results for Site A, high concentration plume, bioaugmentation treatment C (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of lactate; ⬇ = addition of B_{12}; ⬇ = addition of SRB.

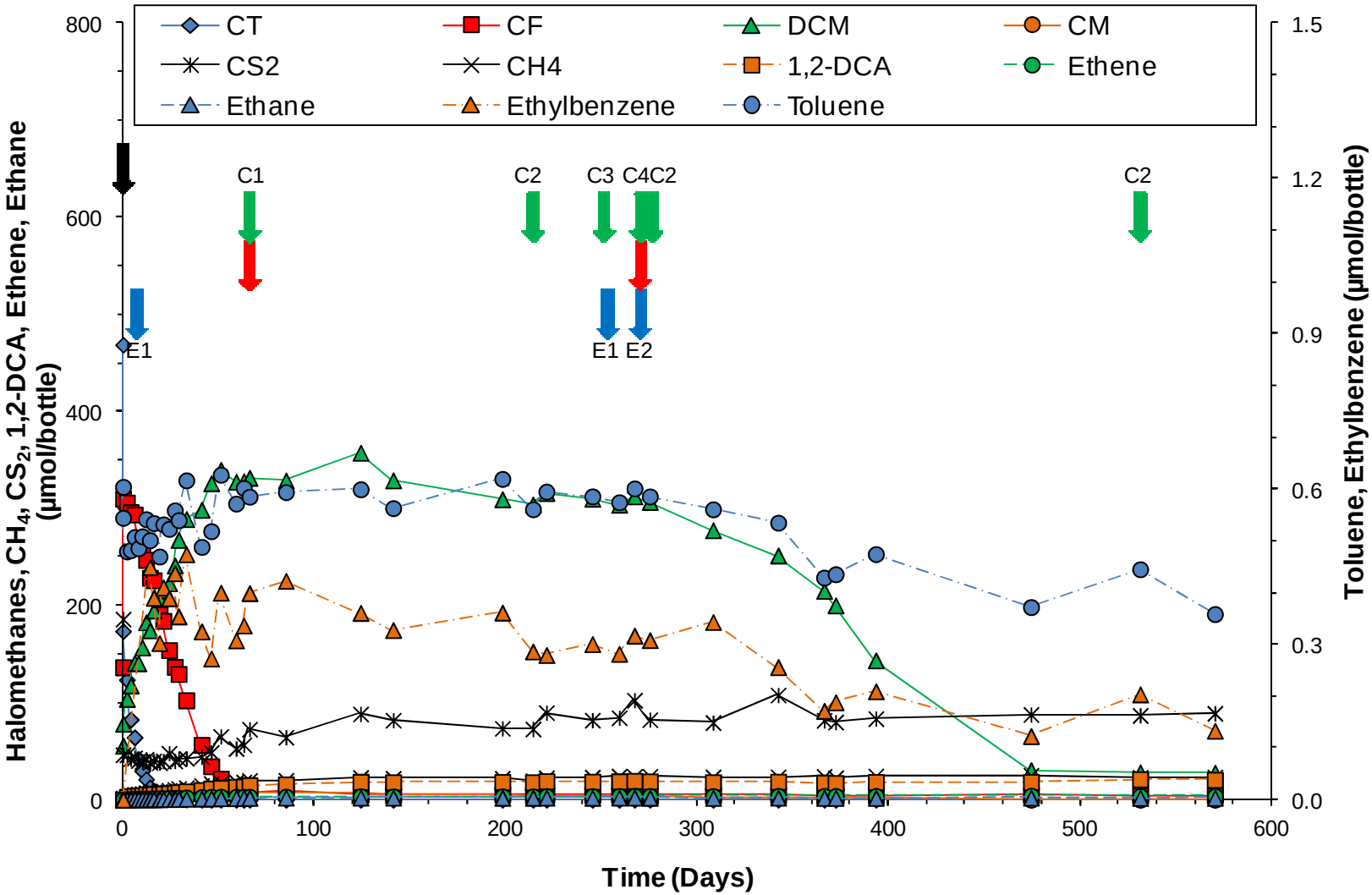

Figure 4.9 Representative results for Site A, high concentration plume, ZVI + bioaugmentation treatment (bottle #1); ⬇ = addition of ZVI; ⬇ = addition of electron donor (E1 = lactate, E2 = corn syrup); ⬇ = addition of B_{12}; ⬇ = addition of cultures (C1 = SDC-9, C2 = DCM, C3 = 1,2-DCA respiring culture, and C4 = DHM-1).

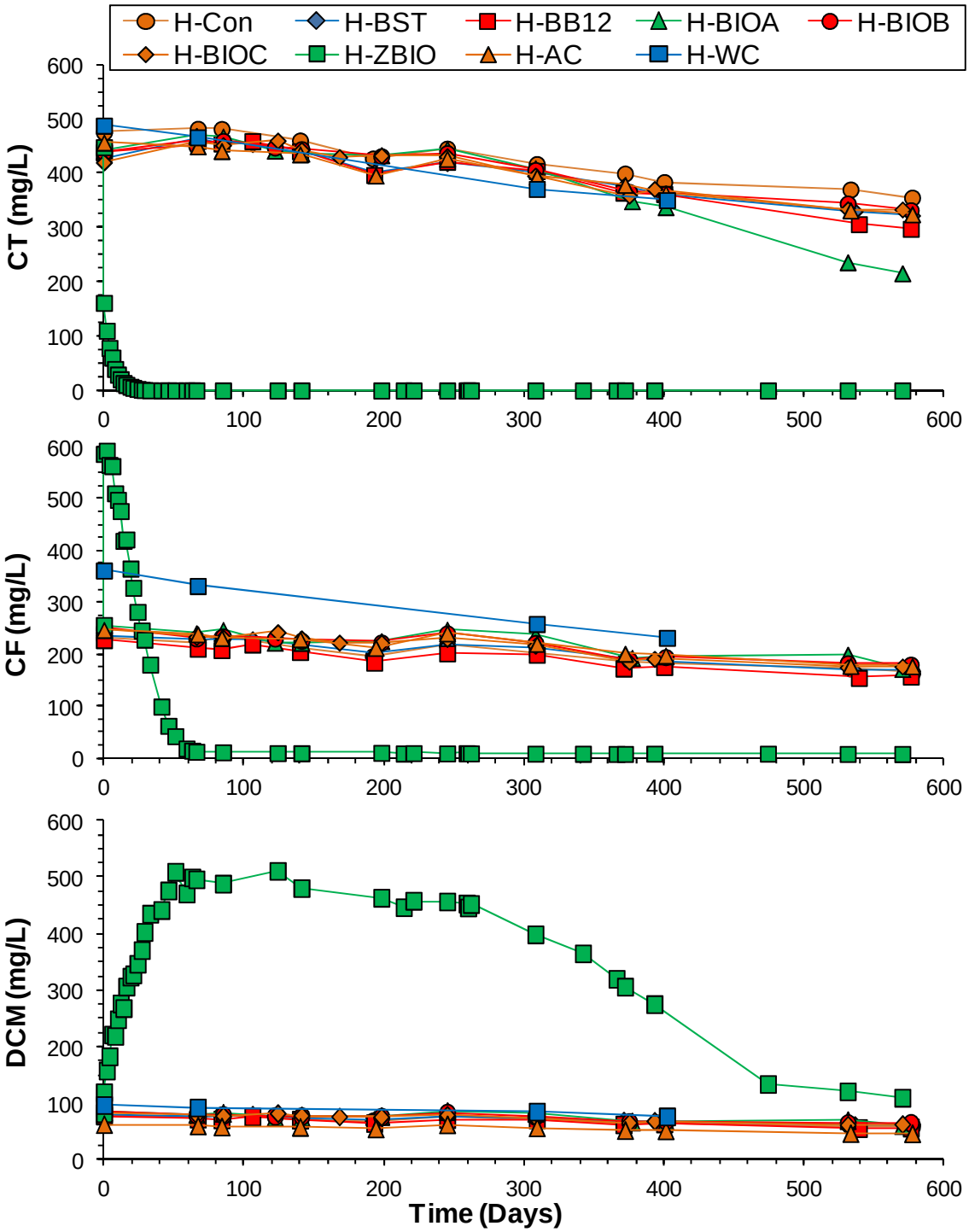

Figure 4.10 Performance of all treatments on CT, CF and DCM for Site A, high concentration plume; Con = unamended control; BST = biostimulation; BB12 = biostimulation + B_{12}; BIOA = bioaugmentation with DHM-1; BIOB = bioaugmentation with SDC-9; BIOC = bioaugmentation with SRB; ZBIO = ZVI + bioaugmentation; AC = autoclaved control; WC = water control.

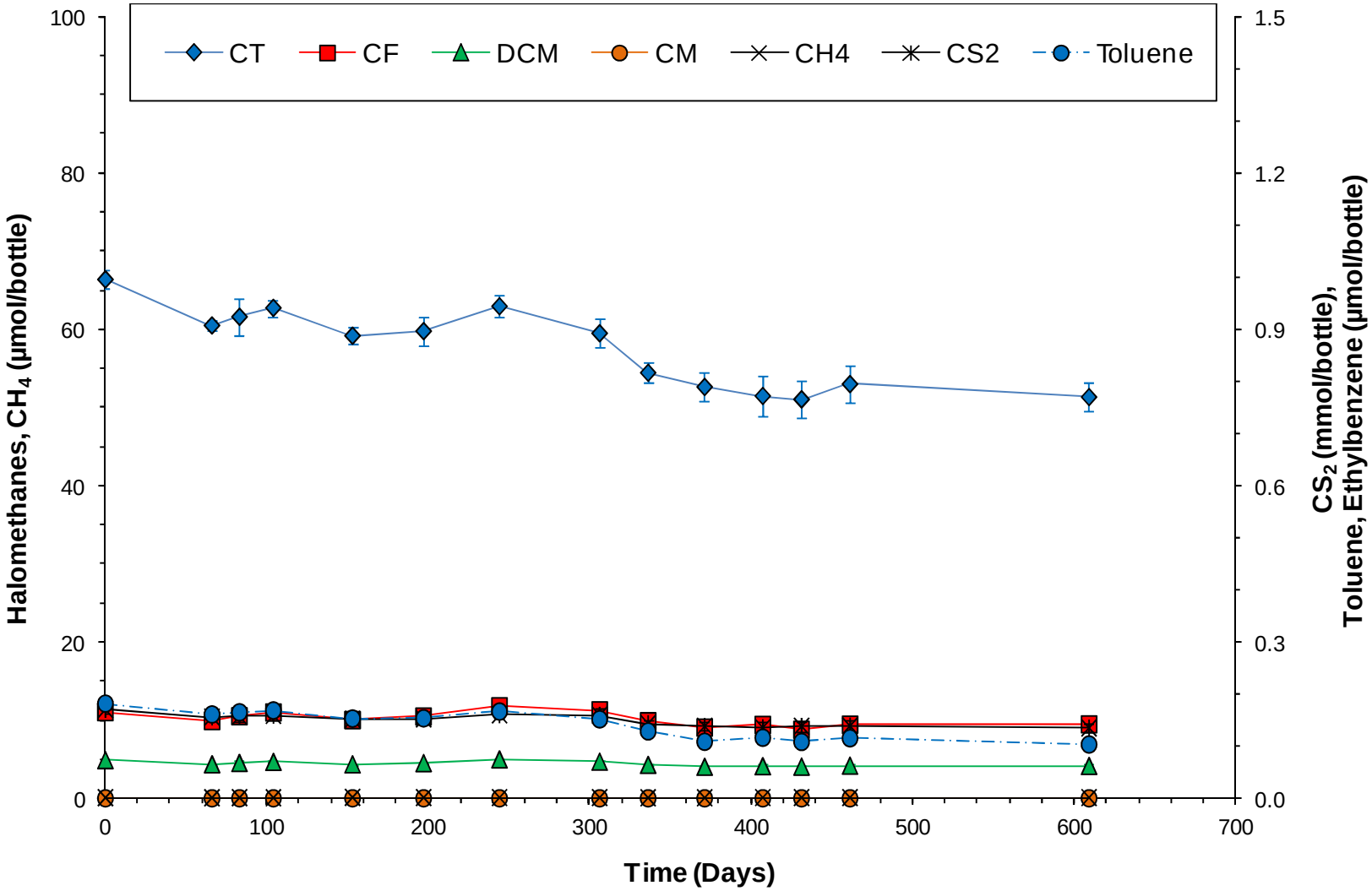

Figure 4.11 Results for Site A, medium concentration plume, autoclaved control treatment (average of triplicate microcosms; error bars indicate one standard deviation).

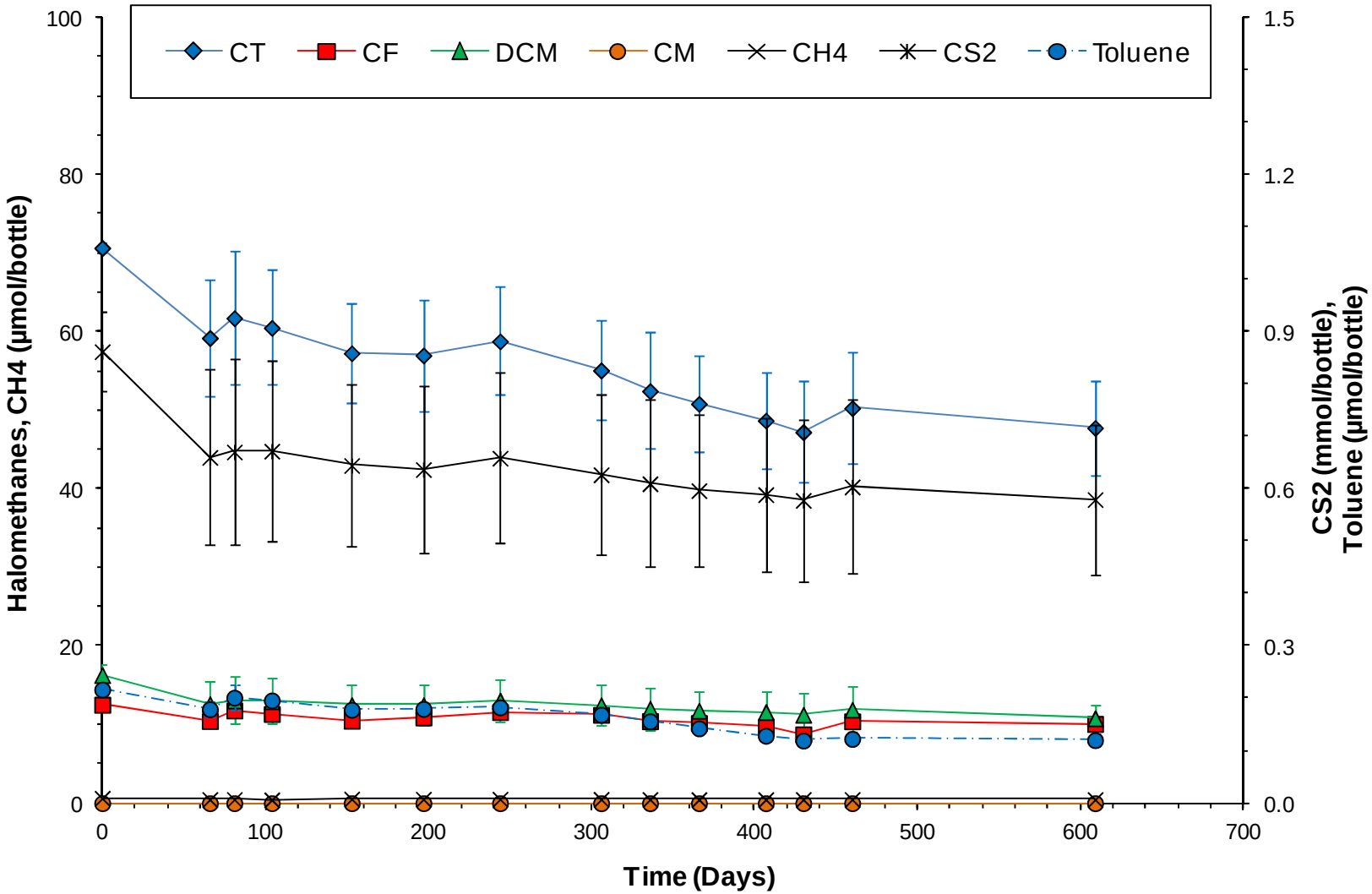

Figure 4.12 Results for Site A, medium concentration plume, unamended treatment (average of triplicate microcosms; error bars indicate one standard deviation).

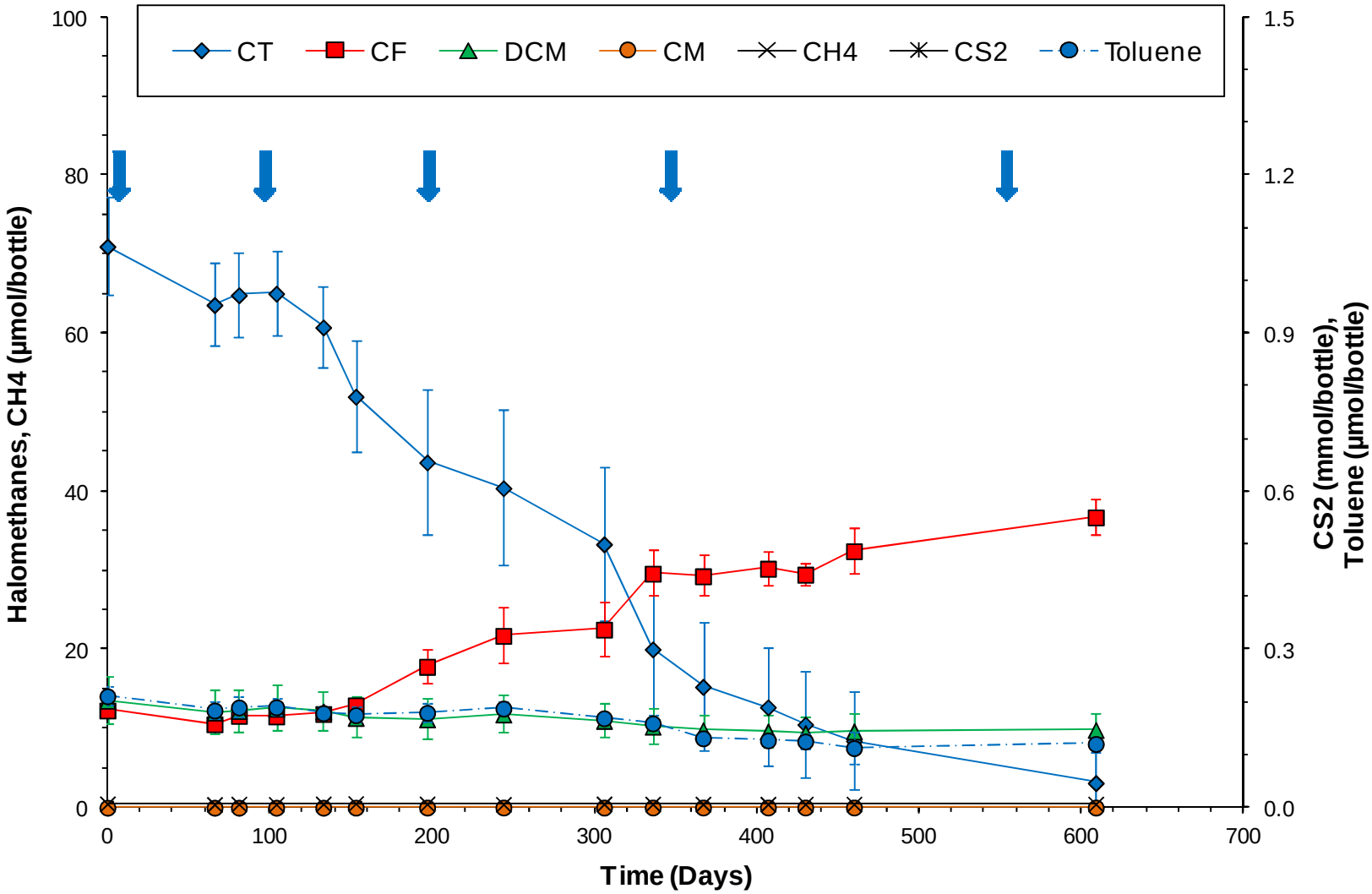

Figure 4.13 Results for Site A, medium concentration plume, biostimulation with corn syrup (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of corn syrup.

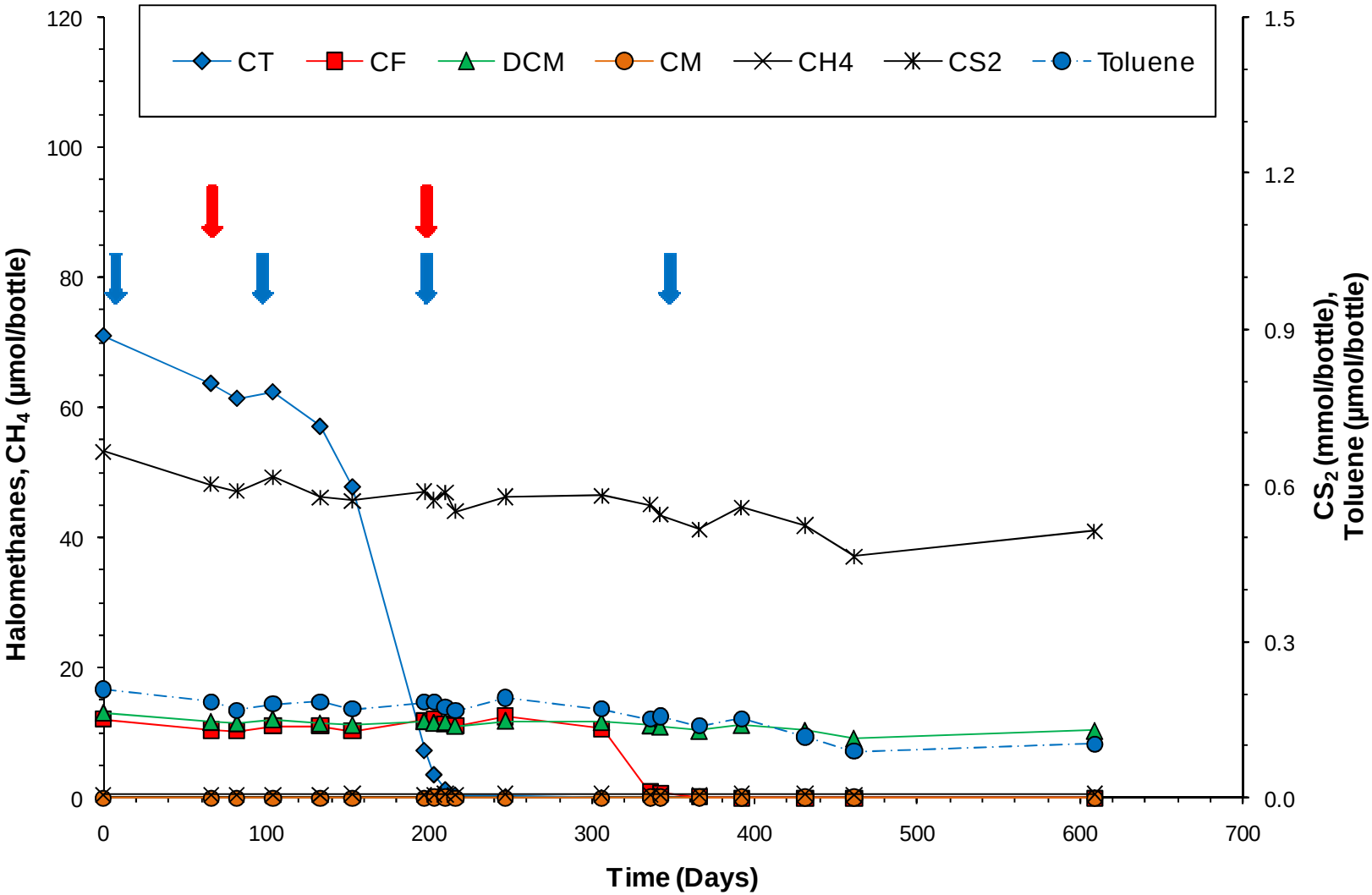

Figure 4.14 Representative results for Site A, medium concentration plume, biostimulation with corn syrup + B_{12} (bottle #2); ↓ = addition of corn syrup; ↓ = addition of B_{12}.

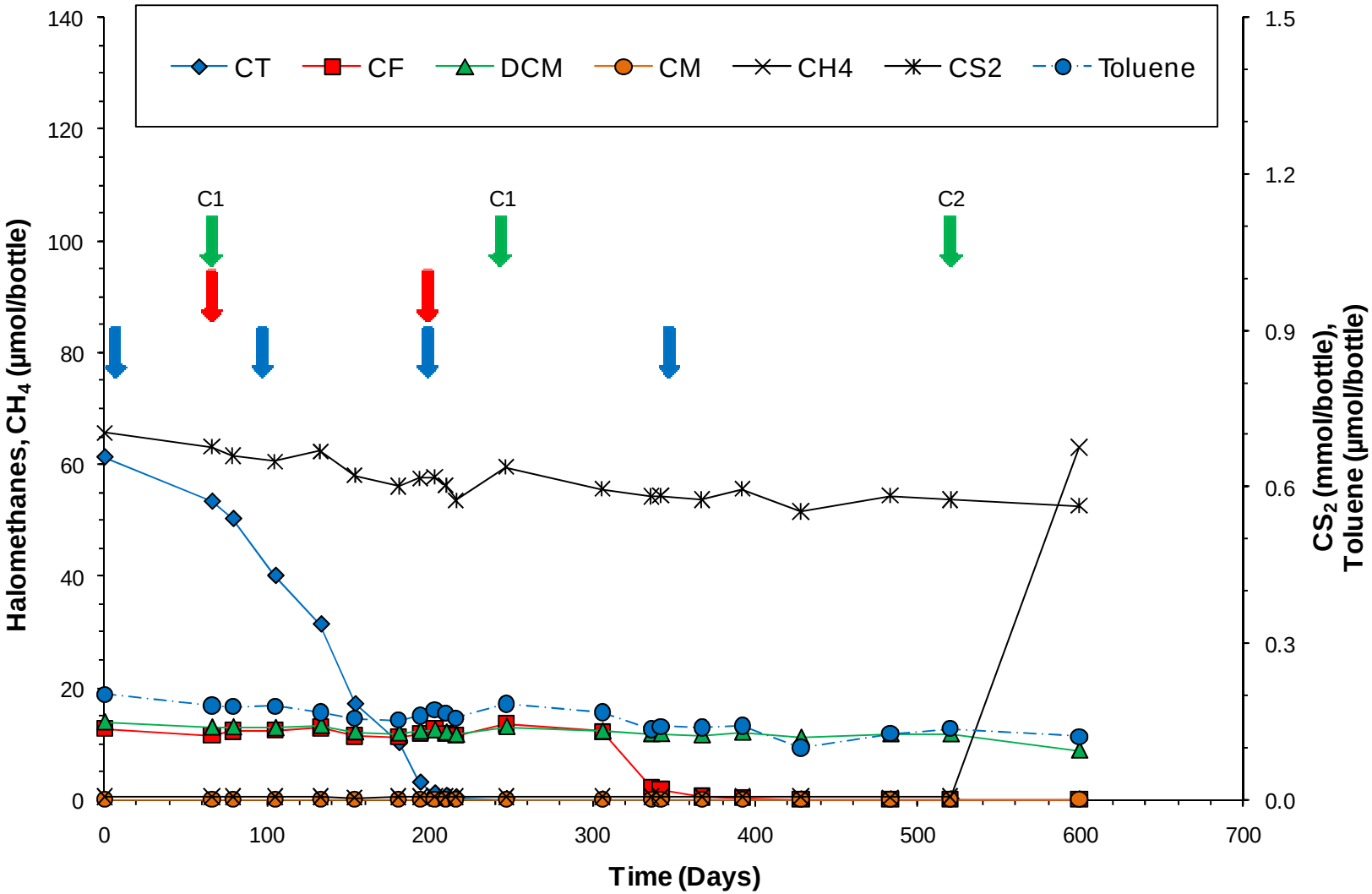

Figure 4.15 Representative results for Site A, medium concentration plume, bioaugmentation treatment A (bottle #2); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of cultures (C1 = DHM-1, C2 = DCM).

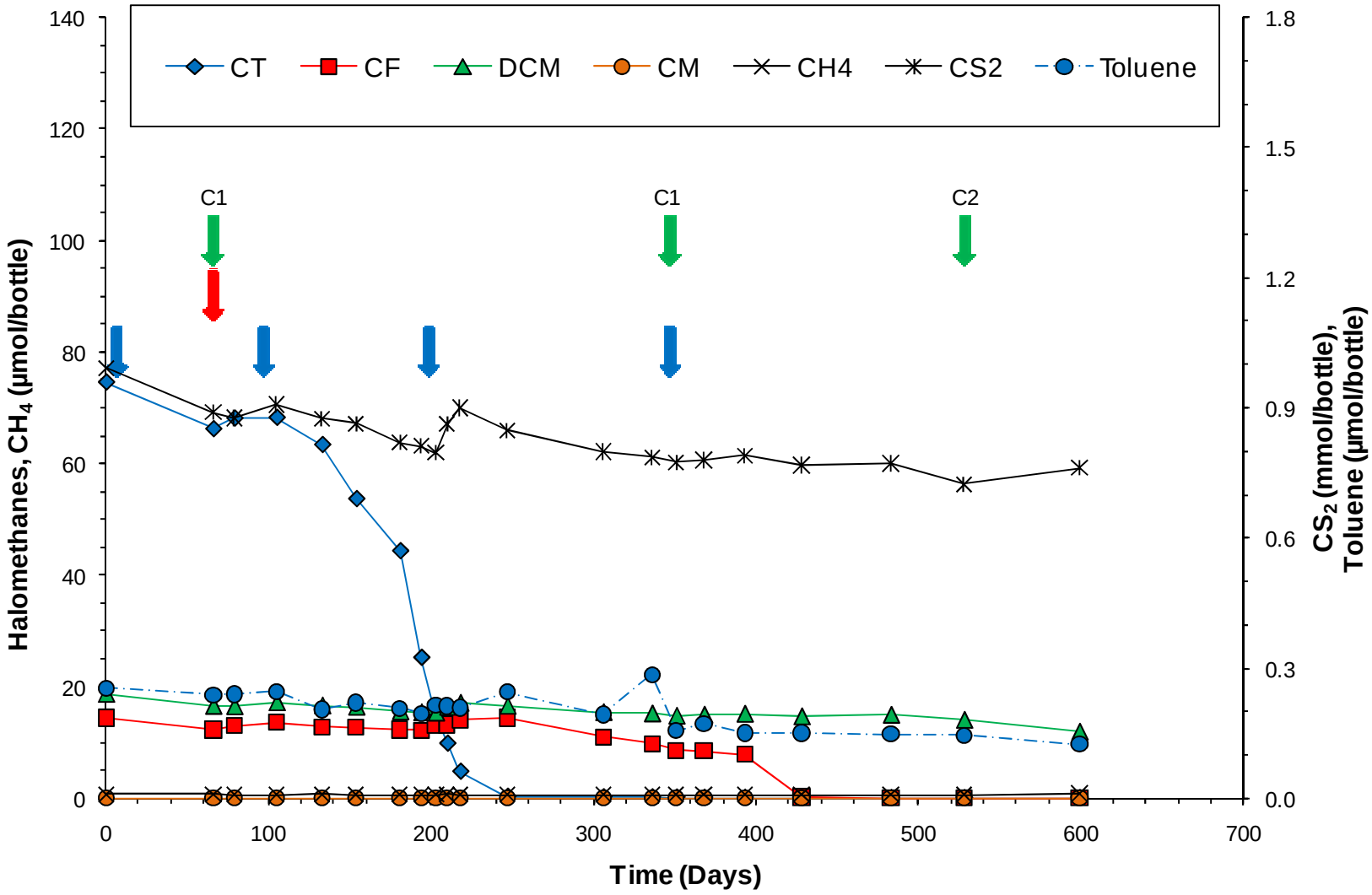

Figure 4.16 Representative results for Site A, medium concentration plume, bioaugmentation treatment B (bottle #2); ↓ = addition of lactate; ↓ = addition of B_{12}; ↓ = addition of cultures (C1 = SDC-9, C2 = DCM).

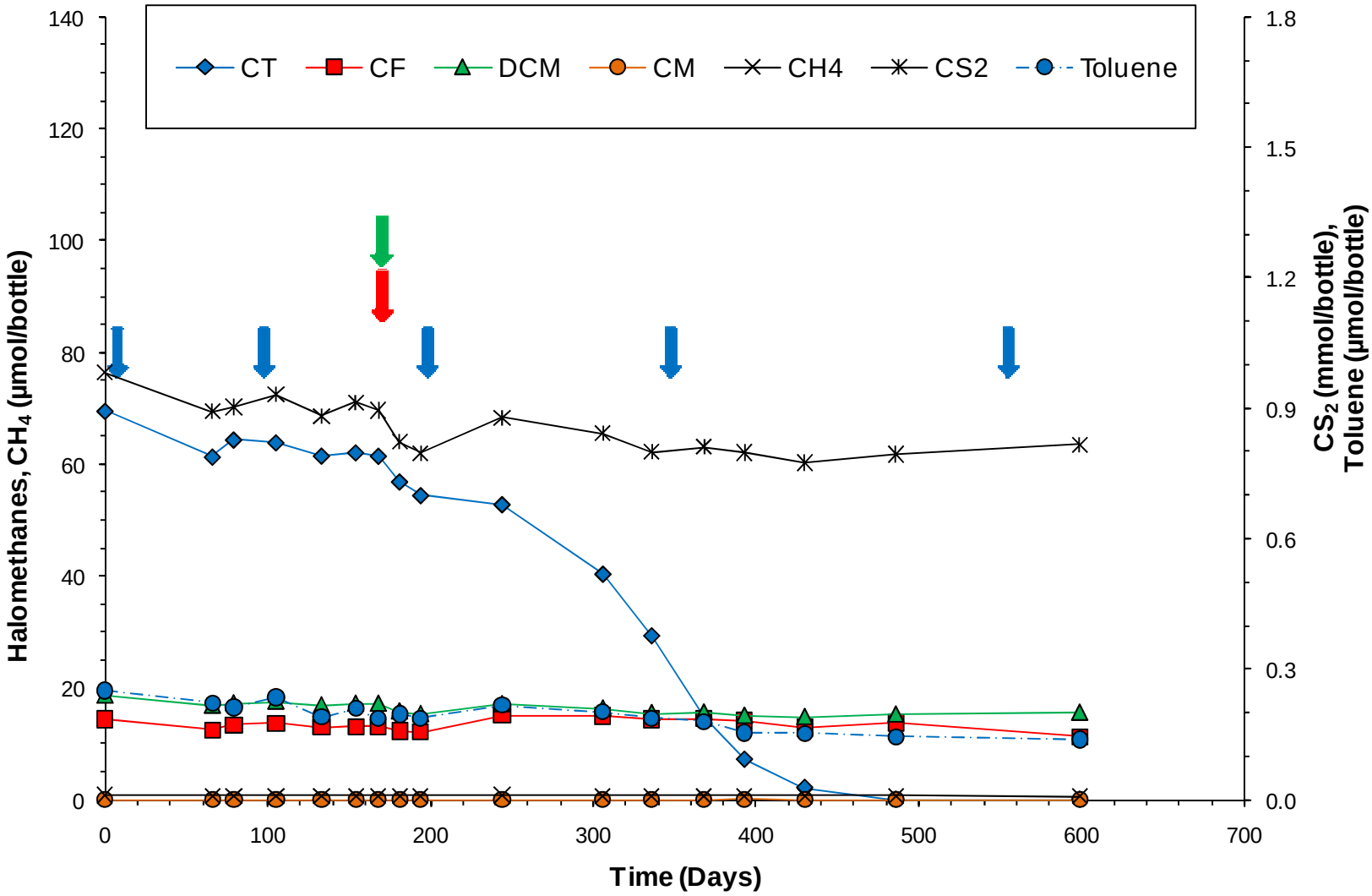

Figure 4.17 Representative results for Site A, medium concentration plume, bioaugmentation treatment C (bottle #1); ↓ = addition of lactate; ↓ = addition of B_{12}; ↓ = addition of SRB.

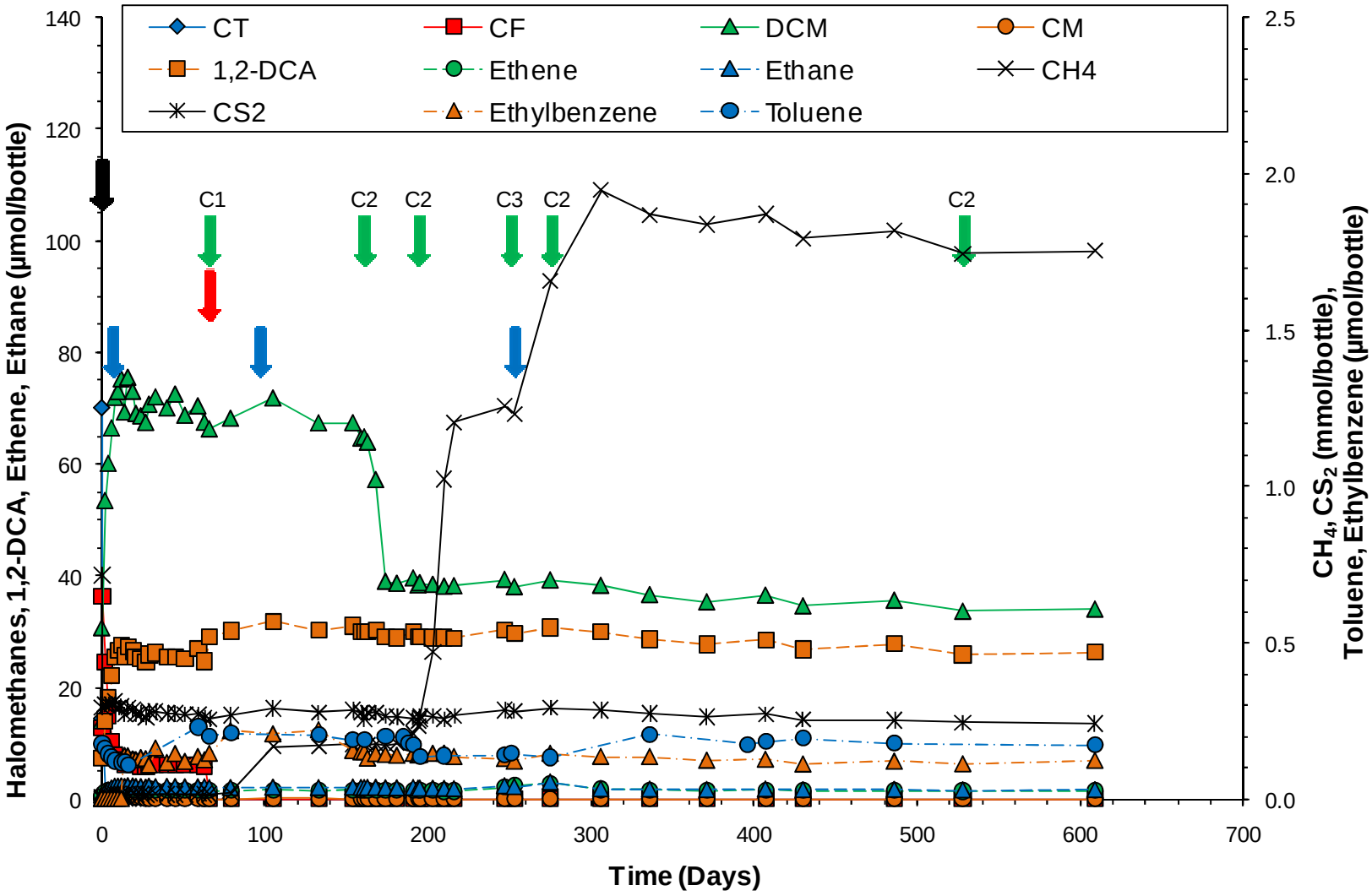

Figure 4.18 Representative results for Site A, medium concentration plume, ZVI + bioaugmentation treatment (bottle #2); ↓ = addition of ZVI; ↓ = addition of lactate; ↓ = addition of B_{12}; ↓ = addition of cultures (C1 = SDC-9, C2 = DCM, and C3 = DCA respiring culture).

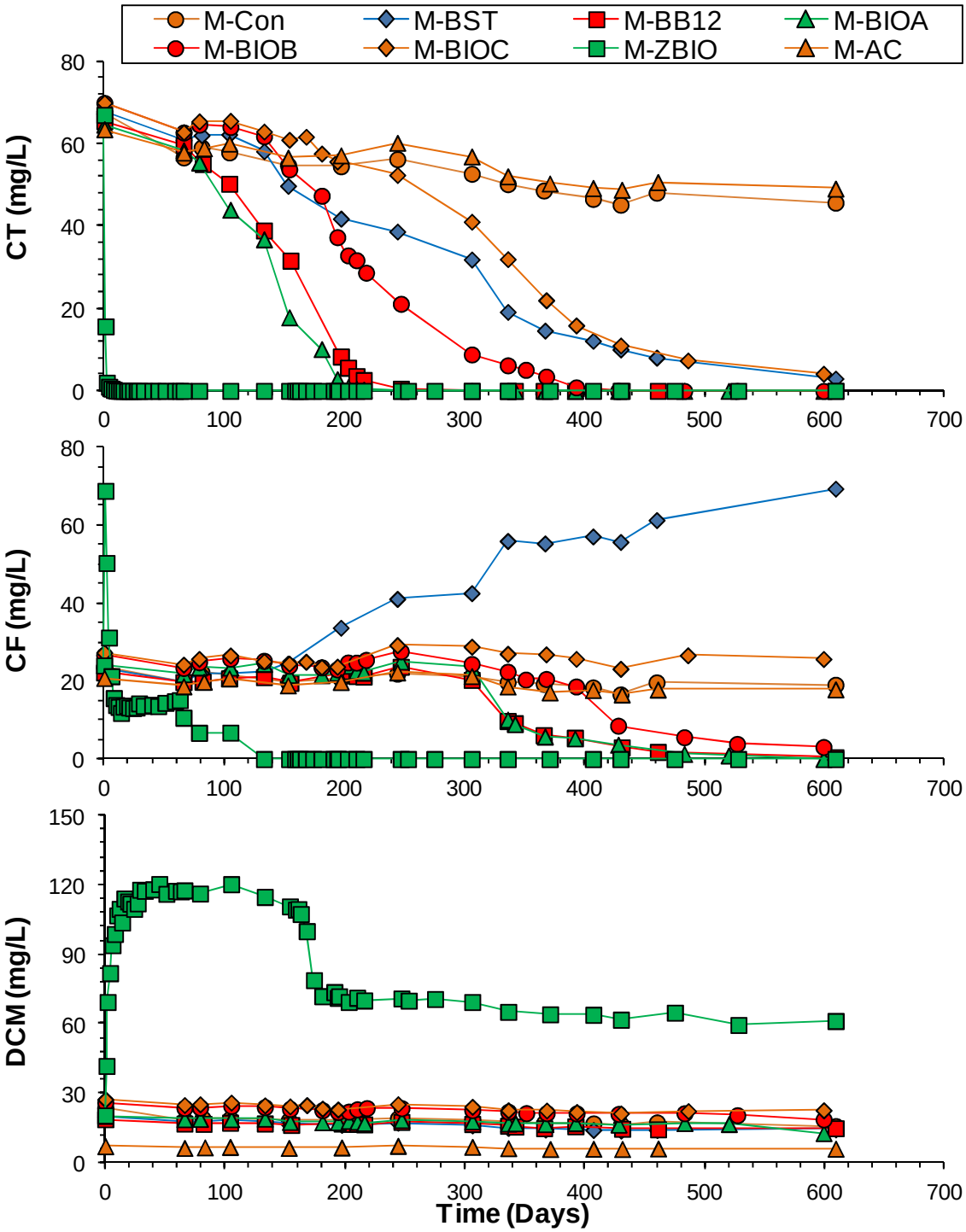

Figure 4.19 Performance of all treatments on CT, CF and DCM for Site A, medium concentration plume; Con = unamended control; BST = biostimulation; BB12 = biostimulation + B_{12}; BIOA = bioaugmentation with DHM-1; BIOB = bioaugmentation with SDC-9; BIOC = bioaugmentation with SRB; ZBIO = ZVI + bioaugmentation; AC = autoclaved control.

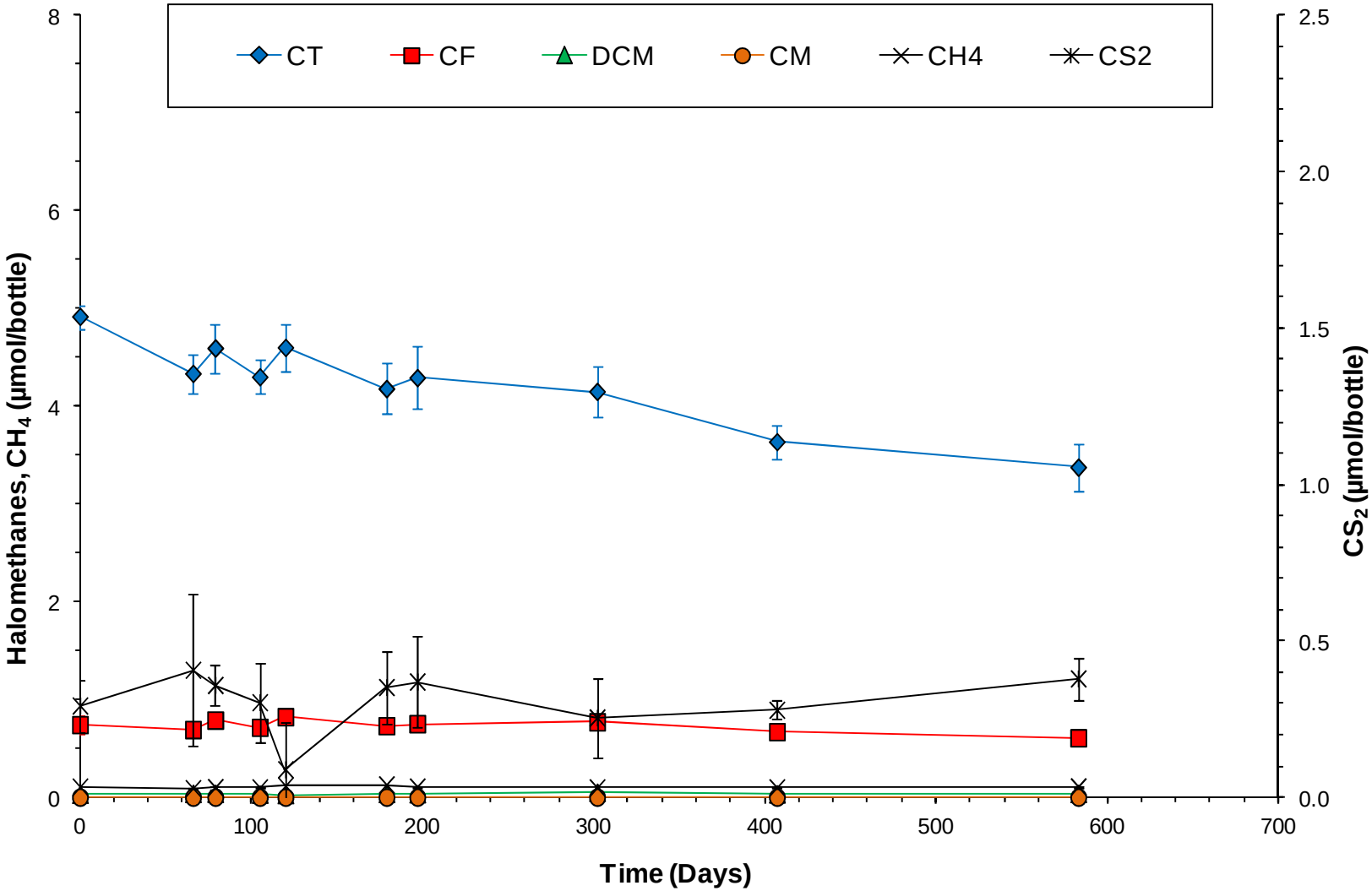

Figure 4.20 Results for Site A, low concentration plume, autoclaved control treatment (average of triplicate microcosms; error bars indicate one standard deviation).

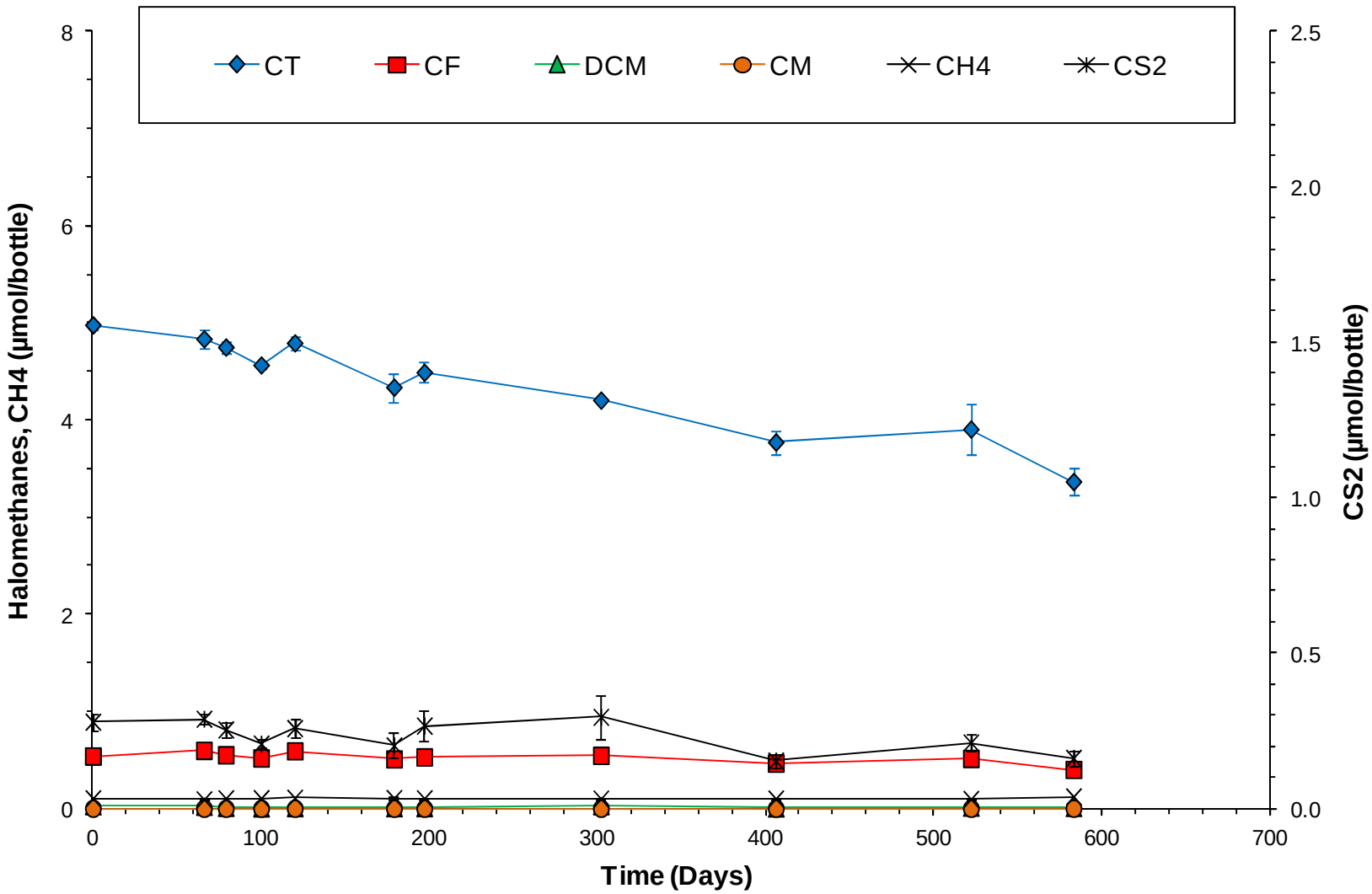

Figure 4.21 Results for Site A, low concentration plume, unamended treatment (average of triplicate microcosms; error bars indicate one standard deviation).

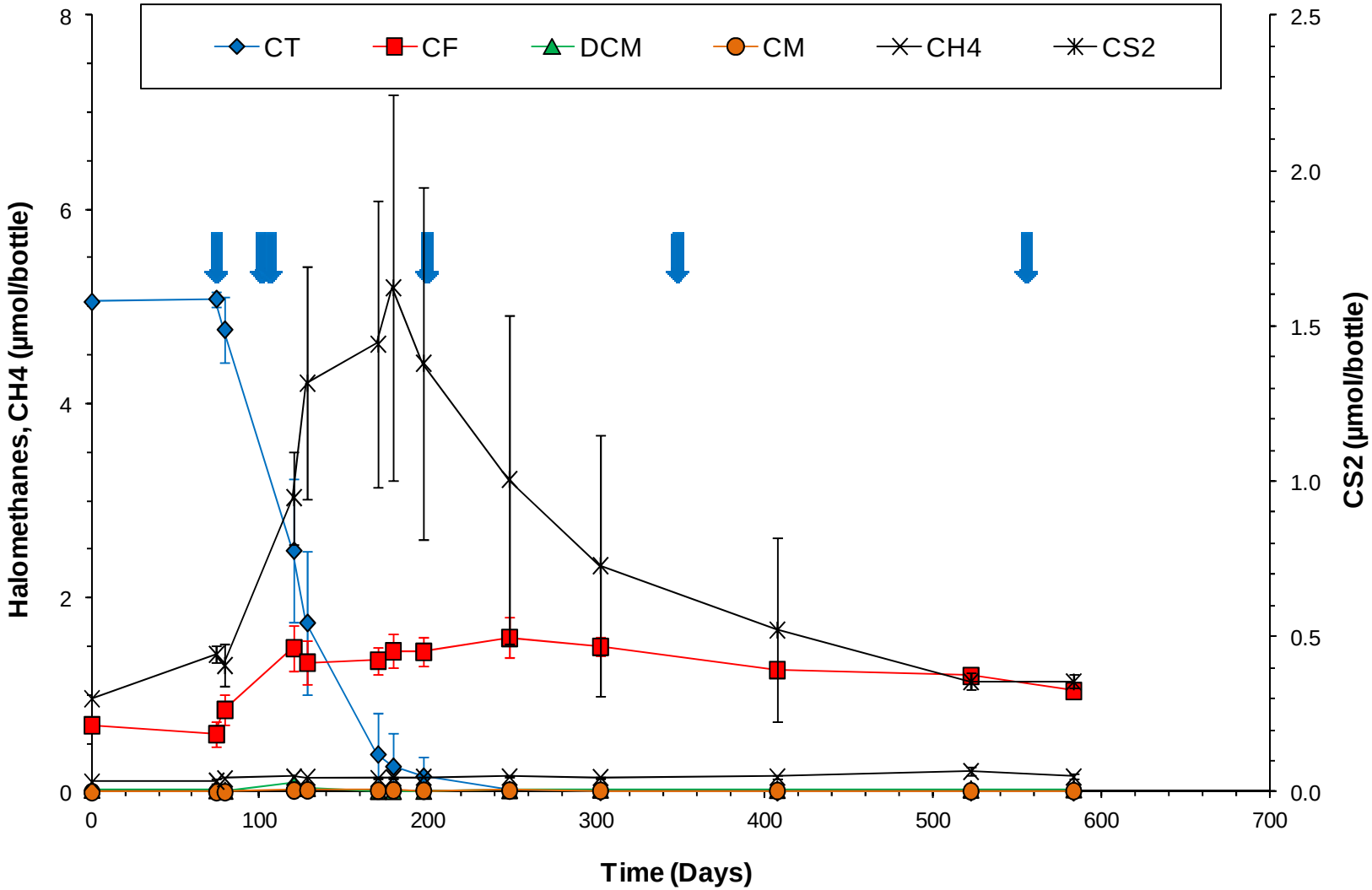

Figure 4.22 Results for Site A, low concentration plume, biostimulation with corn syrup (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of corn syrup.

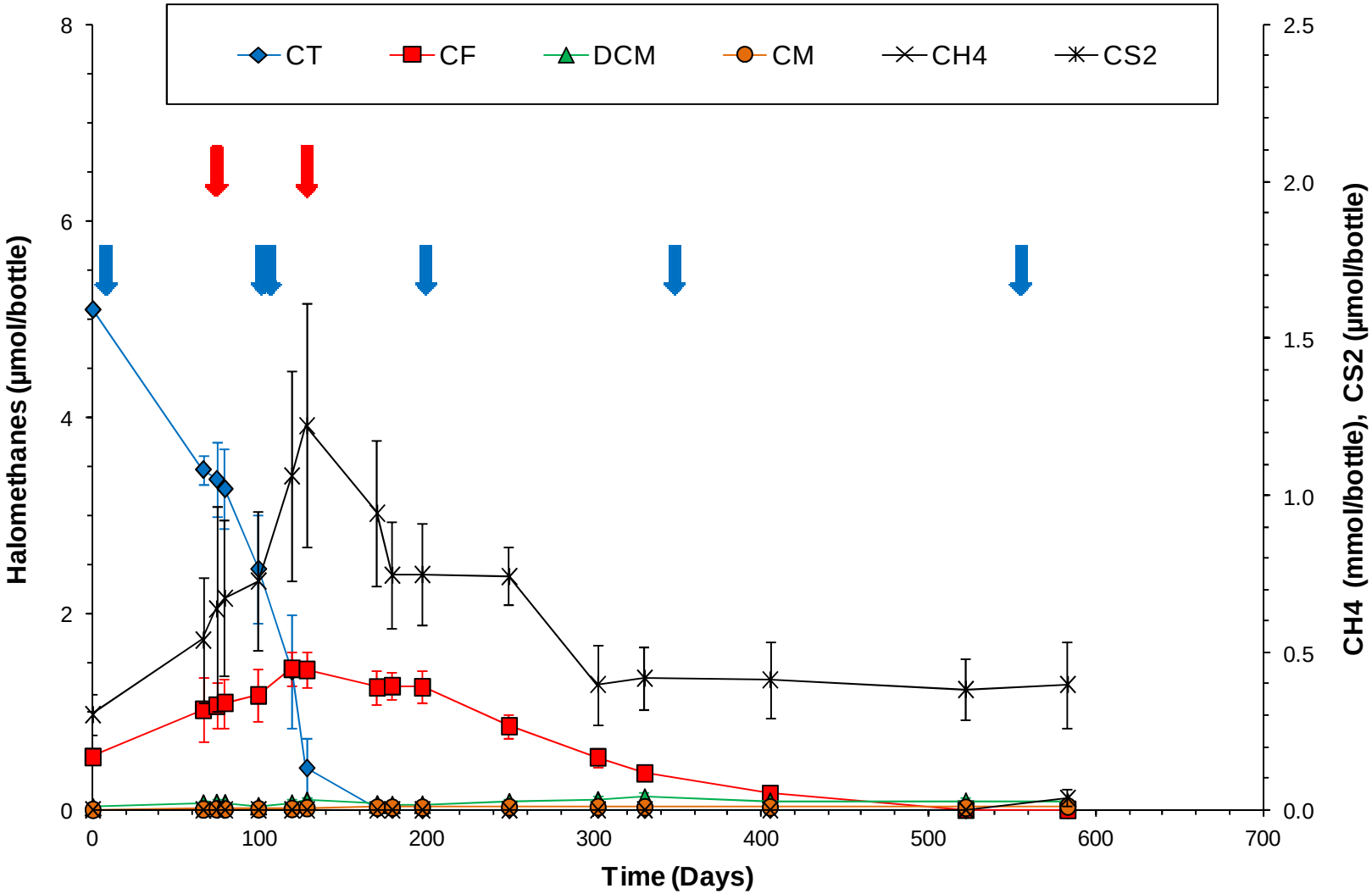

Figure 4.23 Results for Site A, low concentration plume, biostimulation with corn syrup + B_{12} (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of corn syrup; ⬇ = addition of B_{12}.

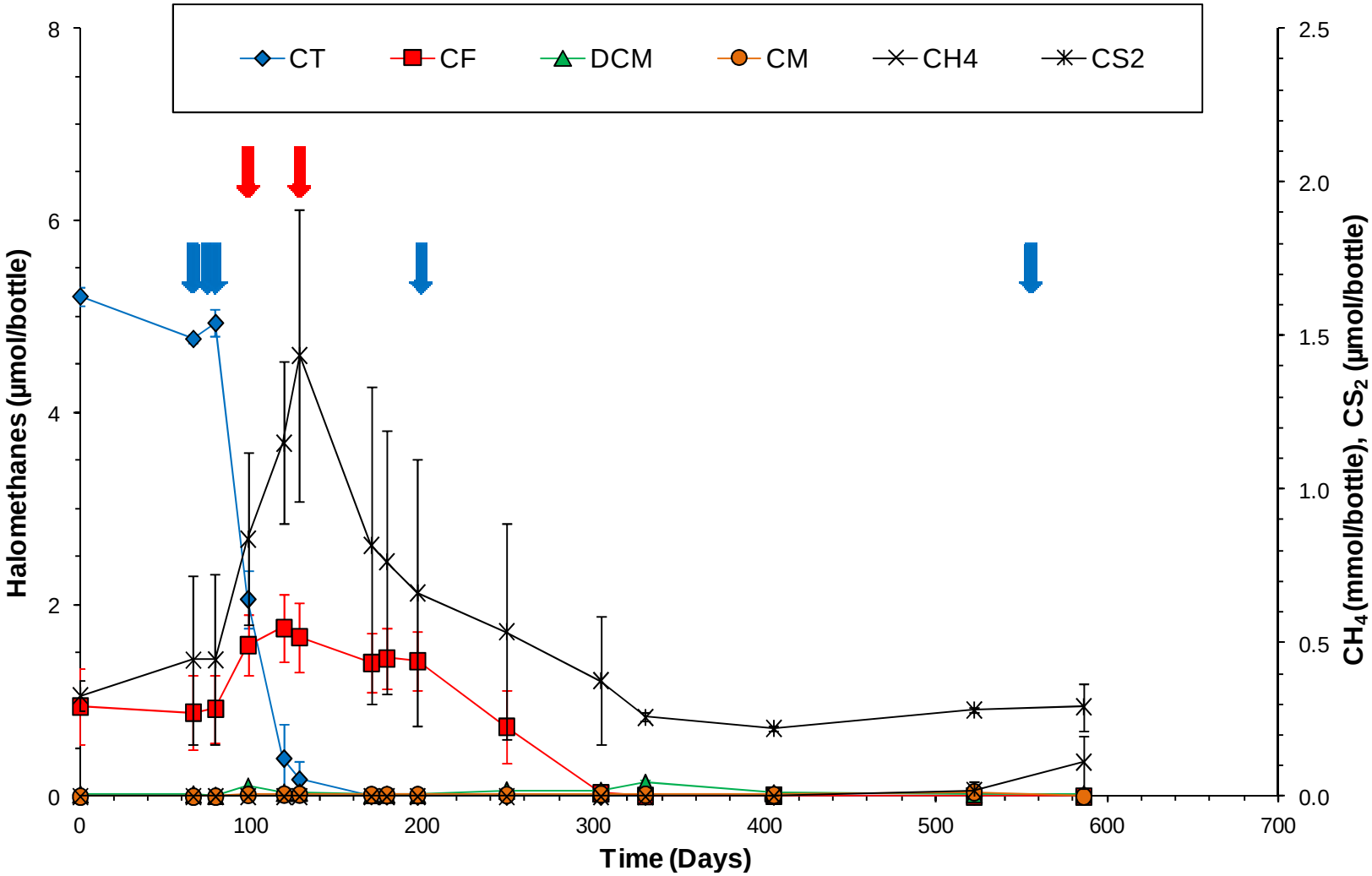

Figure 4.24 Results for Site A, low concentration plume, biostimulation with pH-adjusted groundwater + corn syrup + B_{12} (average of triplicate microcosms; error bars indicate one standard deviation); ⬇ = addition of corn syrup; ⬇ = addition of B_{12}.

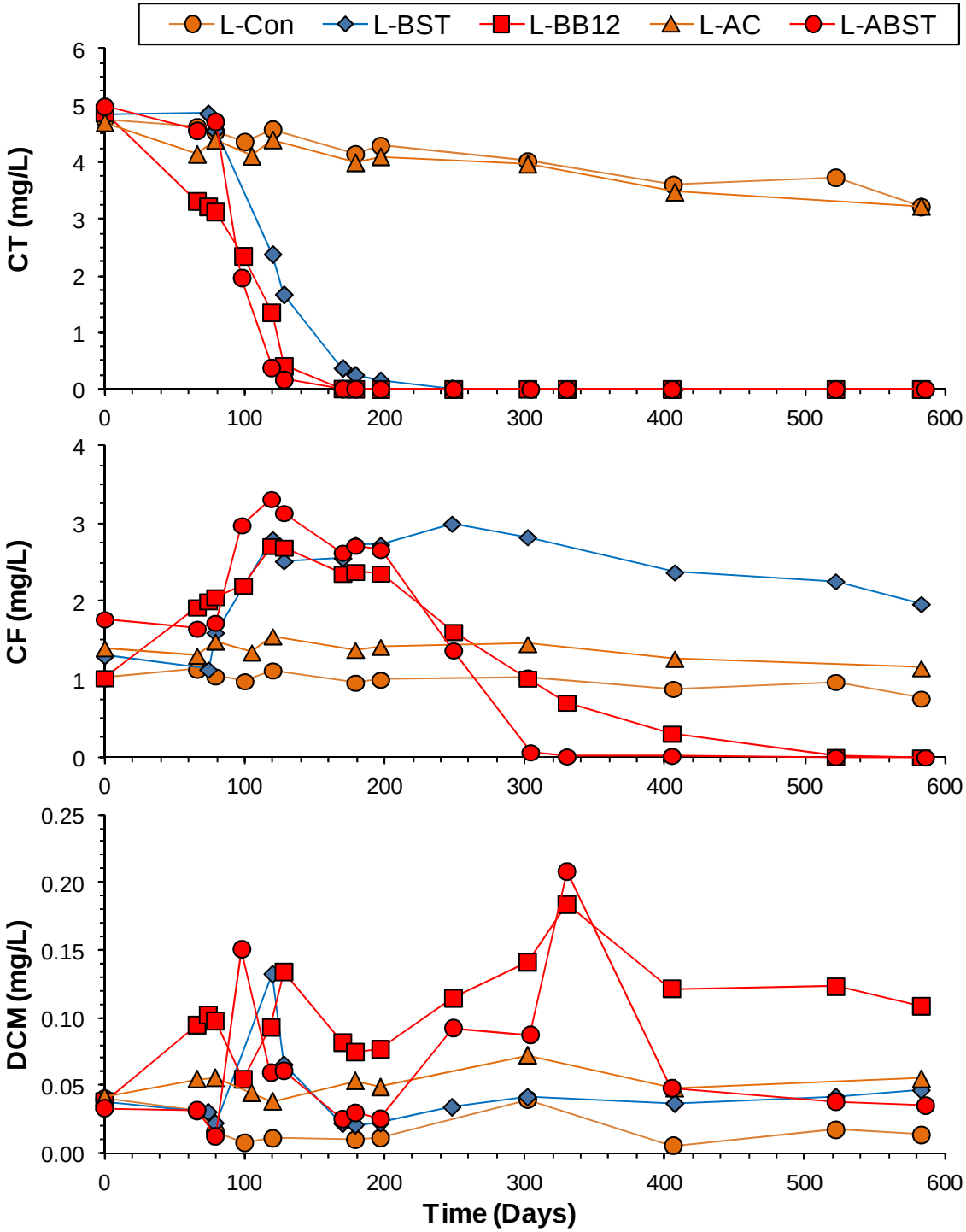

Figure 4.25 Performance of all treatments on CT, CF and DCM for Site A, low concentration plume; Con = unamended control; BST = biostimulation; BB12 = biostimulation + B_{12}; AC = autoclaved control; ABST= biostimulation with ph-adjusted ground water + B_{12}.

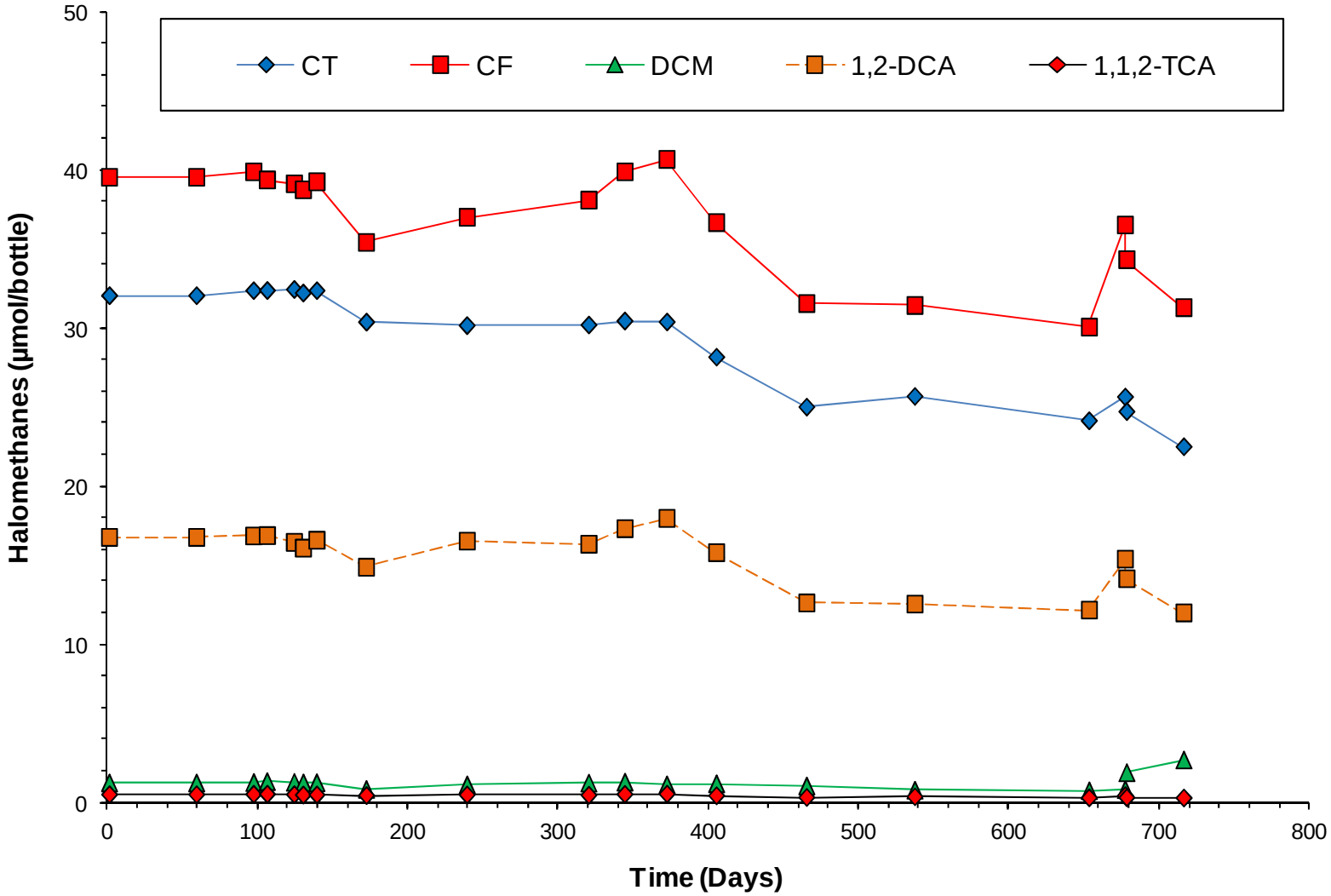

Figure 4.26 Results for Site B, autoclaved control treatment (average of triplicate microcosms; results for individual bottles are provided in Appendix J).

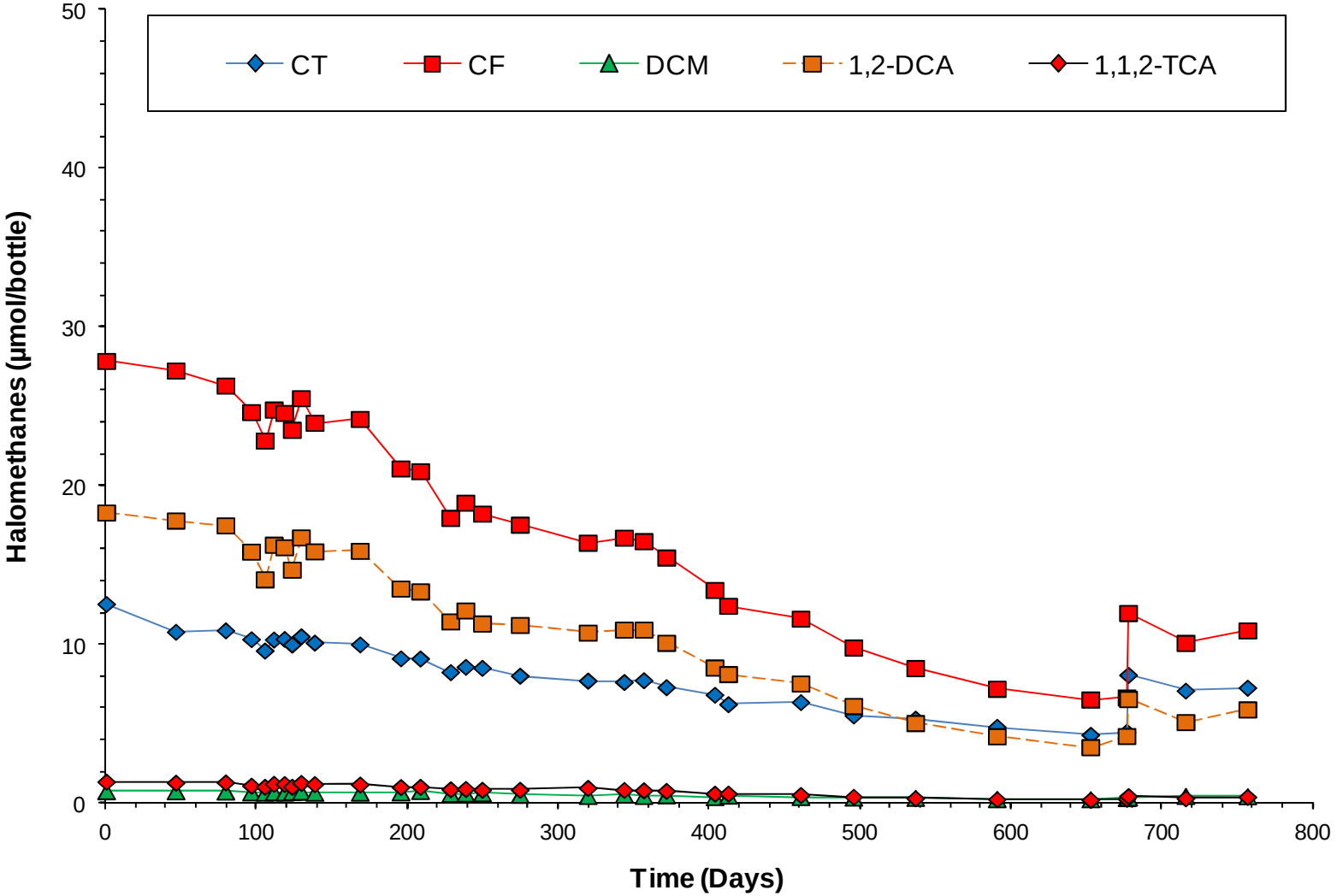

Figure 4.27 Results for Site B, unamended treatment (average of triplicate microcosms; results for individual bottles are provided in Appendix J).

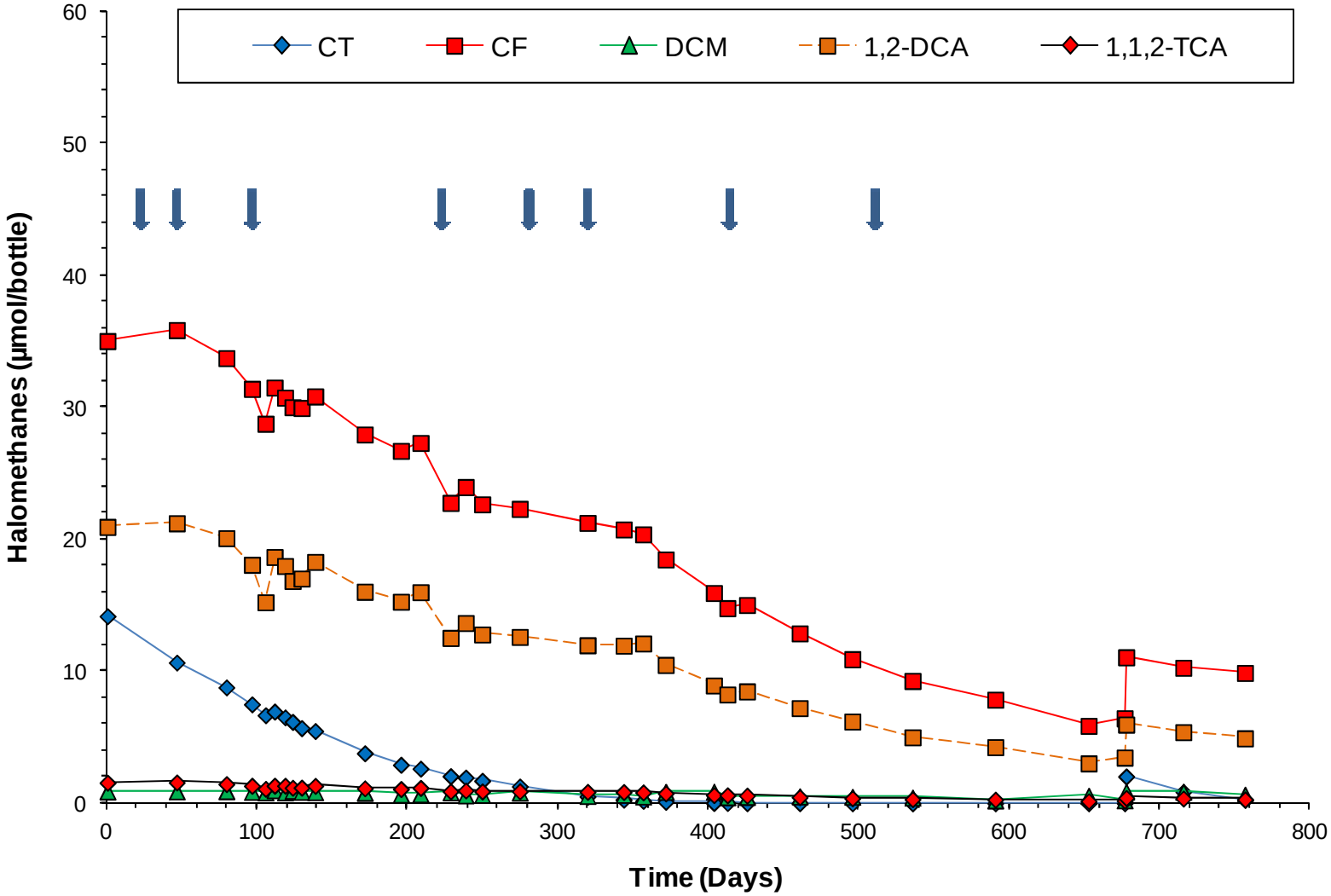

Figure 4.28 Results for Site B, biostimulation with corn syrup (average of triplicate microcosms; results for individual bottles are provided in Appendix J); ⬇ = addition of corn syrup.

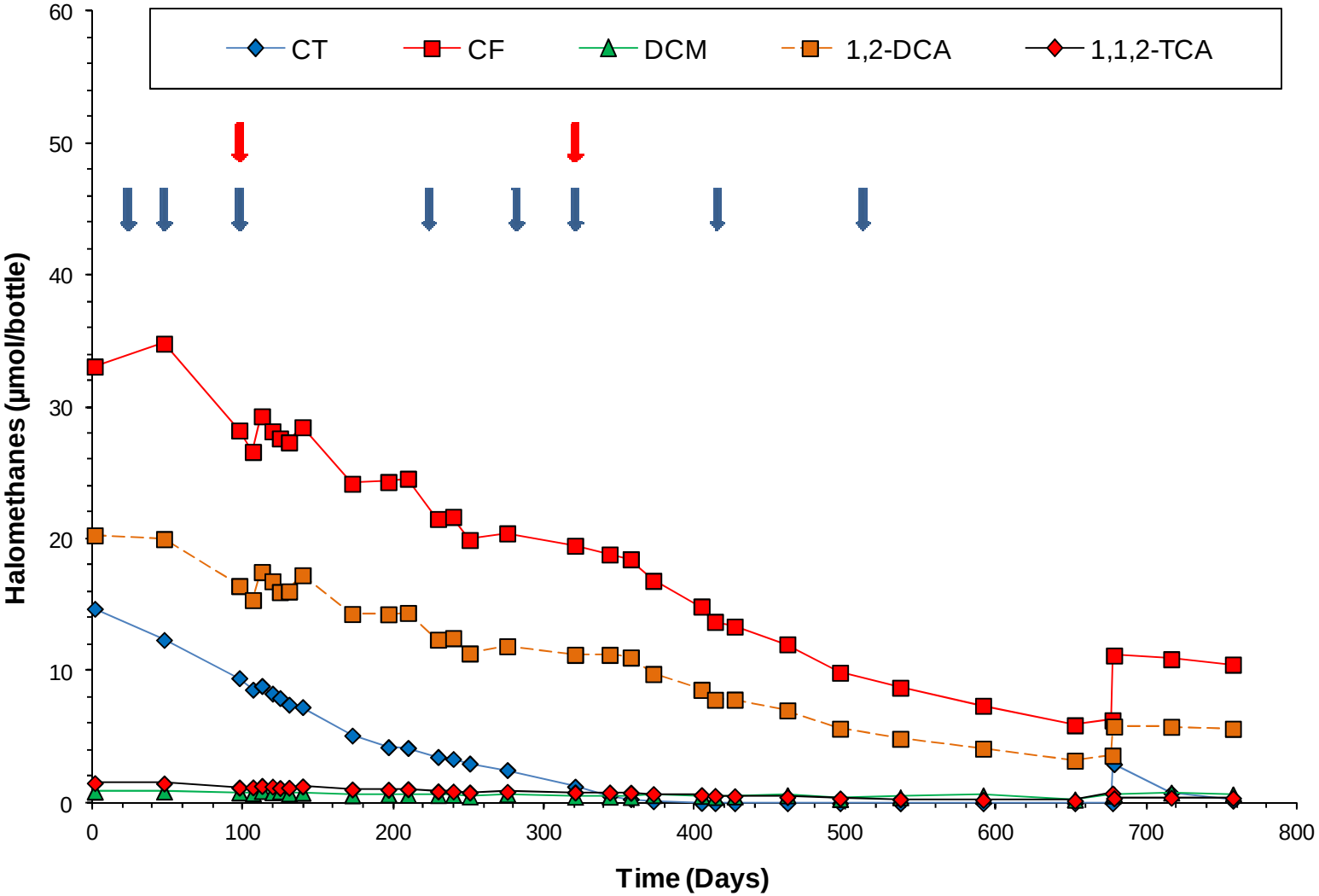

Figure 4.29 Results for Site B, biostimulation with corn syrup + B_{12} (average of triplicate microcosms; results for individual bottles are provided in Appendix J); = addition of corn syrup; = addition of B_{12}.

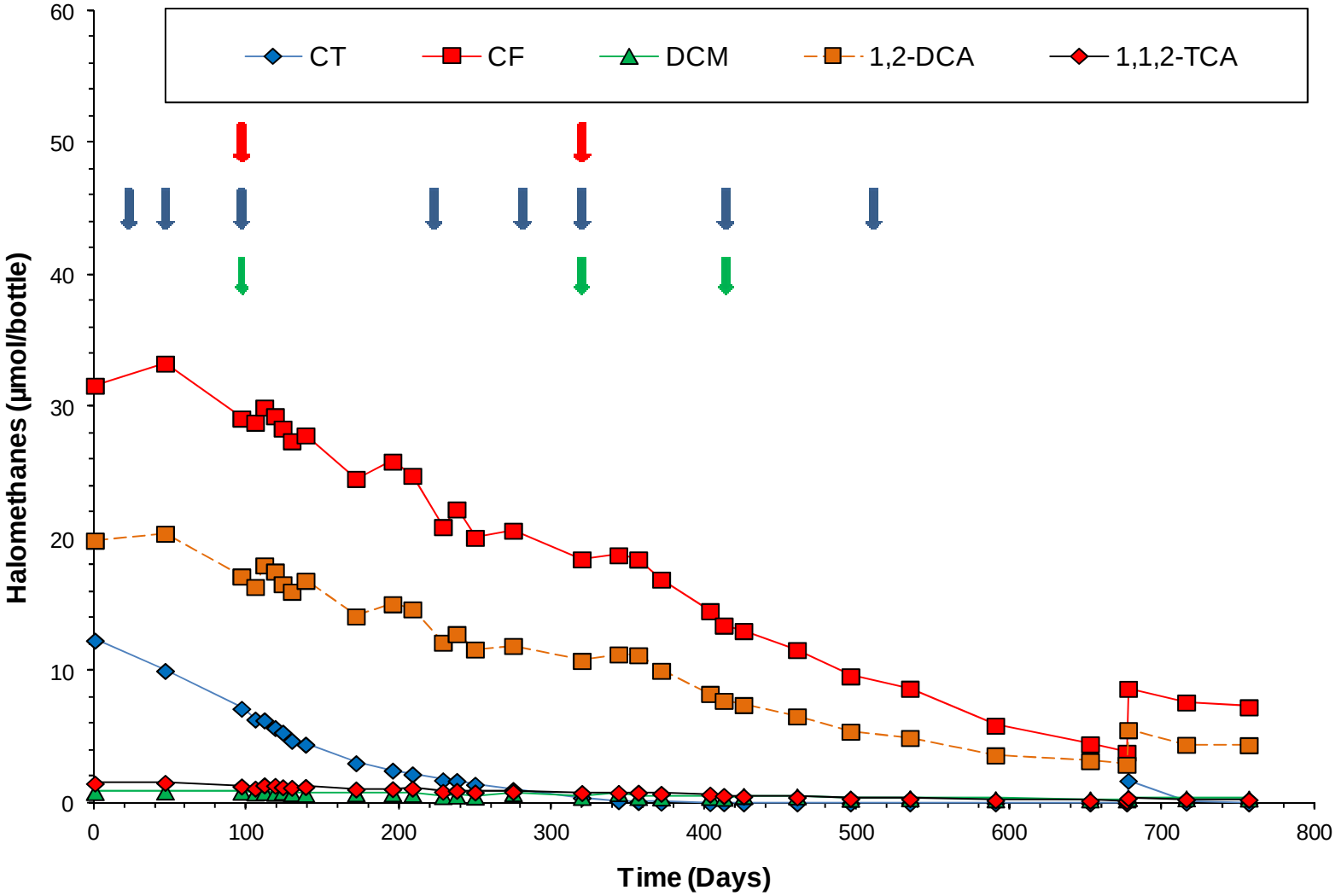

Figure 4.30 Results for Site B, bioaugmentation treatment (average of triplicate microcosms; results for individual bottles are provided in Appendix J); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of DHM-1.

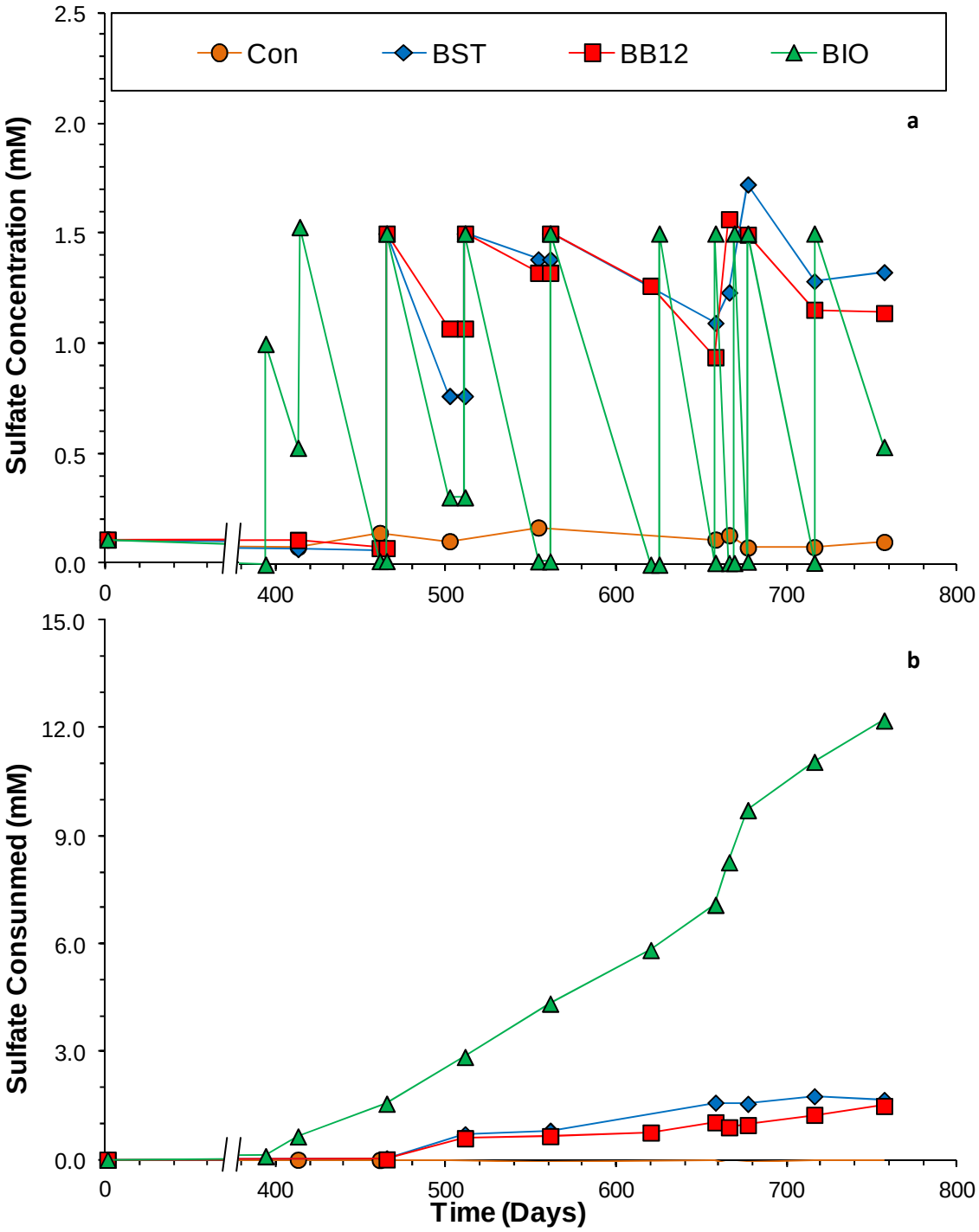

Figure 4.31 Sulfate consumption of all active treatments for Site B (a) for sulfate concentration, and (b) for cumulative sulfate consumption; Con = unamended control; BST = biostimulation; BB12 = biostimulation + B_{12}; BIO = bioaugmentation with DHM-1.

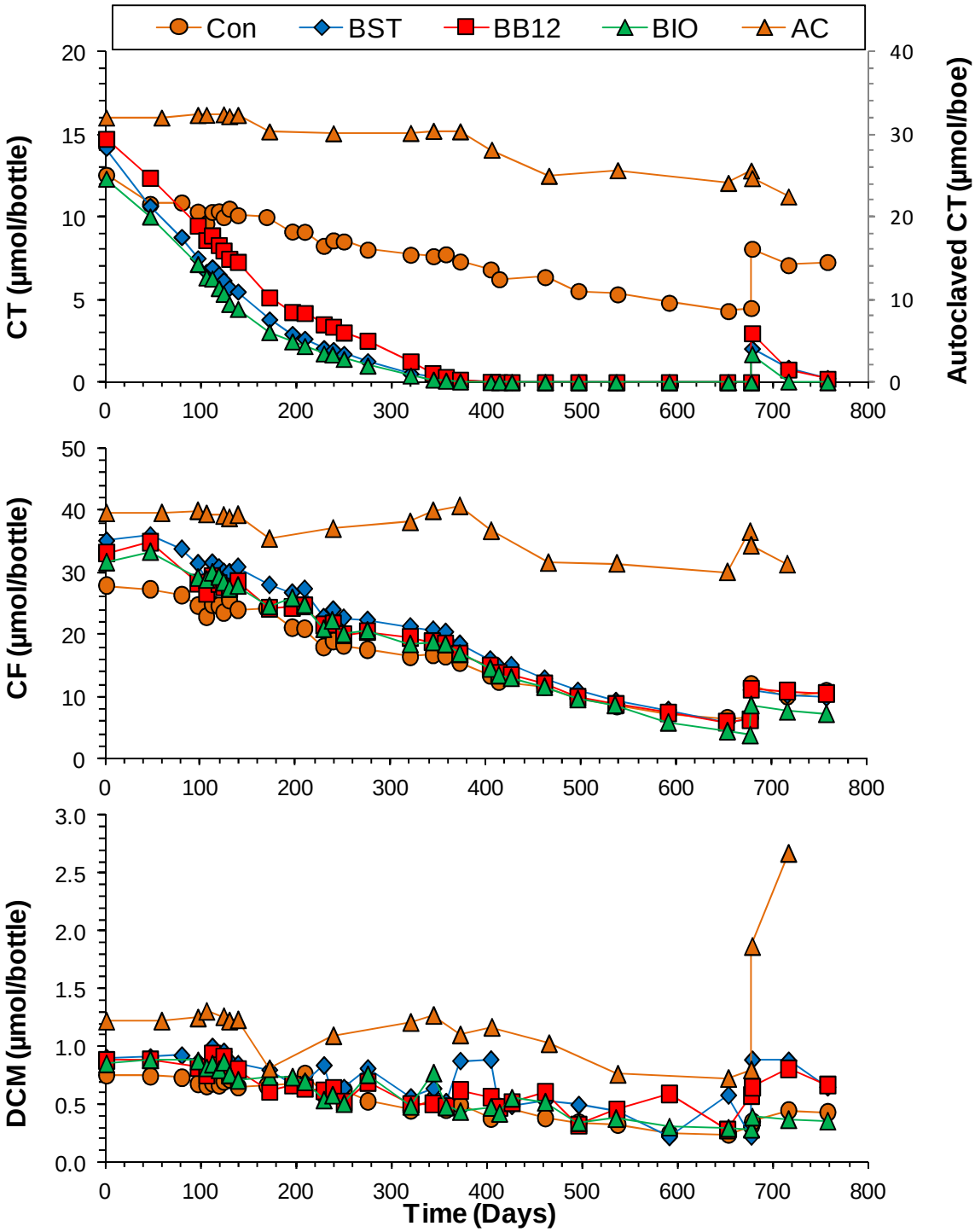

Figure 4.32 Performance of all treatments on CT, CF and DCM for Site B; Con = unamended control; BST = biostimulation; BB12 = biostimulation + B_{12}; BIO = bioaugmentation with DHM-1; AC = autoclaved control.

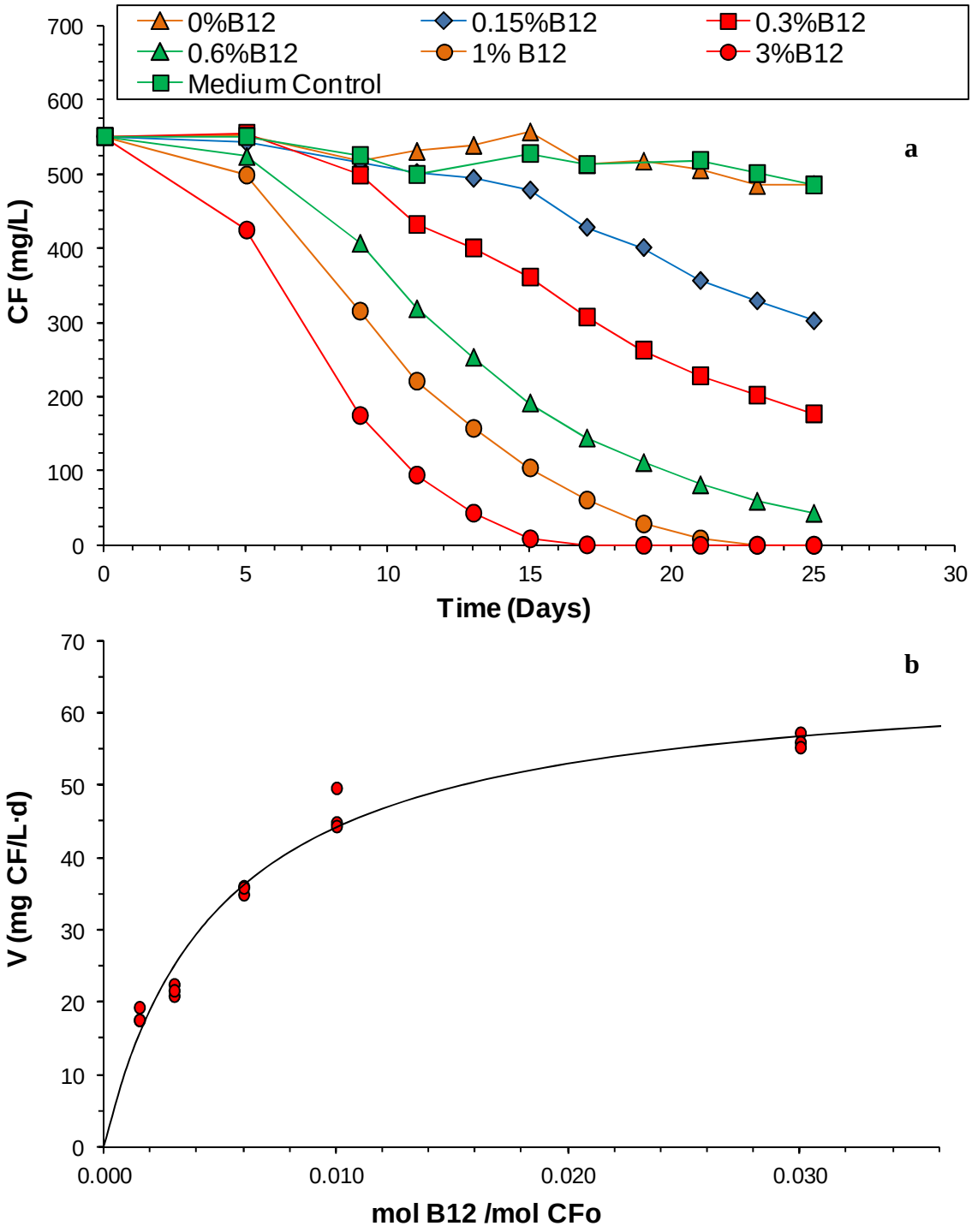

Figure 4.33 The effect of vitamin B_{12} concentration on maximum CF transformation rates by DHM-1 (a) for performances of all treatments with different B_{12} concentrations, and (b) for transformation rate of CF at different B_{12}/CF_o molar ratio.

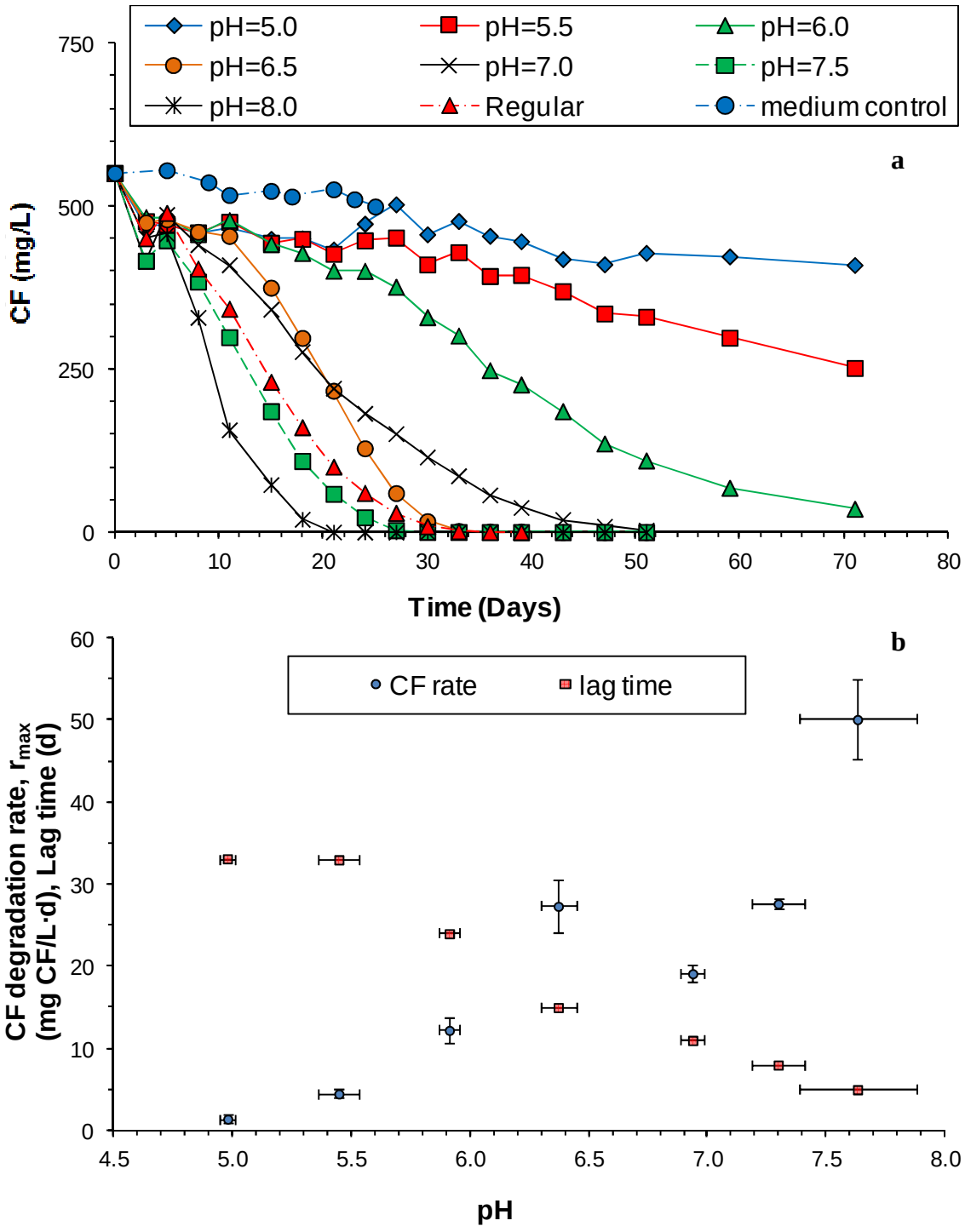

Figure 4.34 Impact of pH on biodegradation rates for CF by DHM-1 and the length of the lag phase prior to the onset of biodegradation. Error bars in both directions indicate the 95% confidence interval, based on results from triplicate bottles at each pH.

APPENDICES

Appendix A GC Standards and Response Factors

Table A.1 GC FID response factors for microcosms with sediment.

Compound	GC RT (min)	Response Factor (µmol/btl/PAU[a])	R^2	Conversion Factor[b] (µmol/btl to µM)	Conversion Factor[b] (µmol/btl to mg/L)
Methane[c]	0.50	3.9810E-06	9.9030E-01	0.00850	0.00014
Ethene	0.68	1.9145E-06	9.9958E-01	1.30419	0.03652
Ethane	0.80	1.7632E-06	9.9961E-01	0.56731	0.01702
CM[c]	1.22	1.2840E-05	9.9610E-01	12.32774	0.62243
DCM[c]	4.60	3.1360E-05	9.9750E-01	17.24790	1.46486
CS_2[c]	5.40	7.4900E-04	9.9490E-01	7.75632	0.59050
CF[c]	7.16	2.4690E-05	9.9910E-01	15.81607	1.88812
1,2-DCA[d]	7.50	2.1618E-05	9.9960E-01	18.06492	1.78770
CT[c]	8.60	8.7800E-06	9.9890E-01	6.21792	0.95644
1,1,2-TCA[d]	10.60	5.0000E-06	-	18.75165	2.50157
Toluene	16.50	1.8000E-06	9.9930E-01	13.21717	1.21785
Ethylbenzene	23.00	1.5644E-06	9.9740E-01	12.67880	1.34623

[a] PAU = peak area units; btl = bottle.
[b] Based on liquid volume of 50 mL and gas volume of 99 mL at 23ºC.
[c] Determined by Shan (38).
[d] Determined by Yu (48).

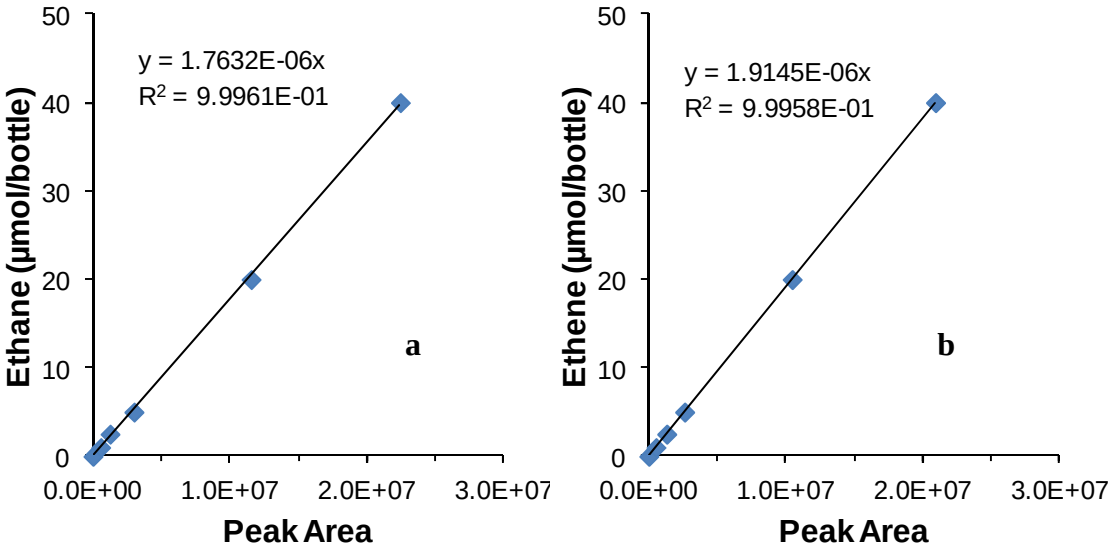

Figure A.1 GC FID response factor curves for ethane (a) and ethene (b); 50 mL DDI water + 11 mL glass beads + 99 mL headspace.

Table A.2 GC FID response factors for microcosms without sediment.

Compound	GC RT (min)	Response Factor (µmol/btl/PAU[a])	Conversion Factor[b] (µmol/btl to mg/L)
CM[c]	1.22	1.2210E-05	0.42480
CS_2	5.40	7.2230E-04	0.51497
DCM	4.60	5.3150E-05	0.81013
CF	7.16	3.3332E-05	1.10520

[a] PAU = peak area units.
[b] Based on liquid volume of 100 mL and gas volume of 60 mL at 23ºC.
[c] Determined by Shan (38).

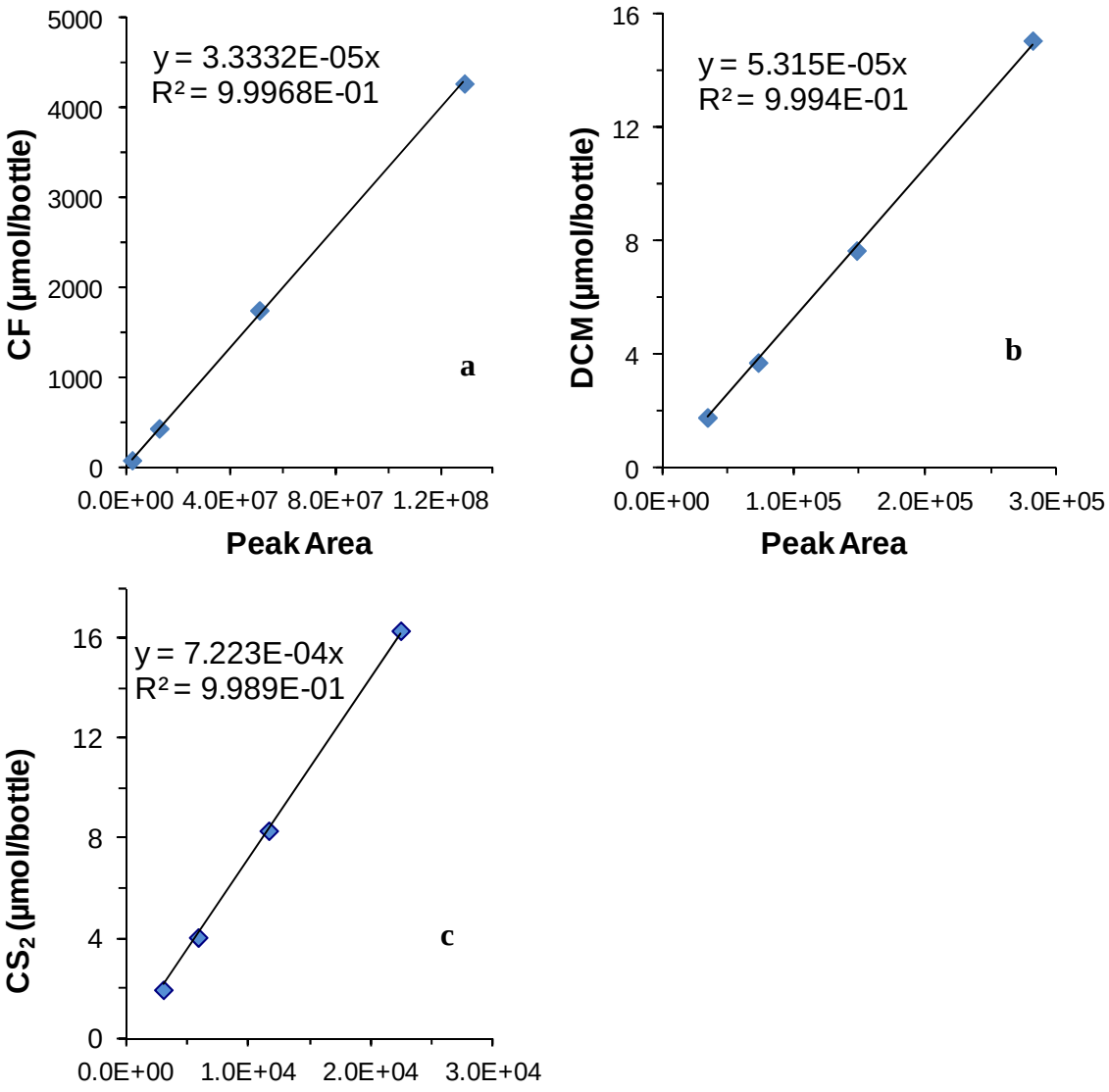

Figure A.2 GC FID response factor curves for CF (a), DCM (b), and CS_2 (c); 100 mL DDI water + 60mL headspace.

Appendix B HPLC Standards and Response Factors

Table B.1 HPLC response factor for acetate, propionate, and lactate on an Aminex® HPX-87H ion exclusion column.

	Acetate		Propionate		Lactate	
Date of Results	Response Factor (mM/PA)	R^2	Response Factor (mM/PA)	R^2	Response Factor (mM/PA)	R^2
10/29/2009	6.197E-06	0.99999	5.066E-06	0.99998	3.031E-06	0.99996
11/4/2009	6.098E-06	0.99999	4.993E-06	0.10000	2.997E-06	0.99999

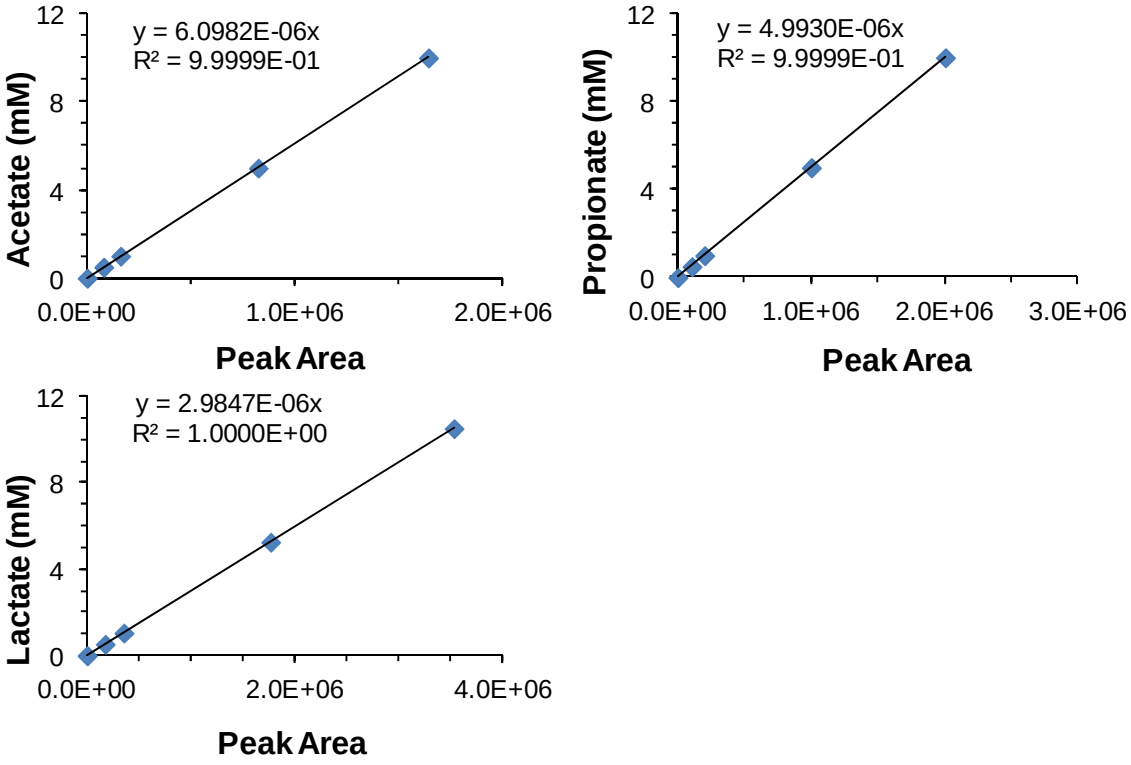

Figure B.1 HPLC response factor curves for acetate (a), propionate (b), and lactate (c) from 11/4/2009.

Appendix C IC Standards and Response Factors

Table C.1 IC response factor for sulfate on Dionex IonPac® AS11 Anion-Exchange Column.

Date of Results	Sulfate Response Factor (mM/PA)	Sulfate R^2
8/18/2010	0.1078	0.9992
9/6/2010	0.1308	0.9909
10/24/2010	0.0923	0.9846

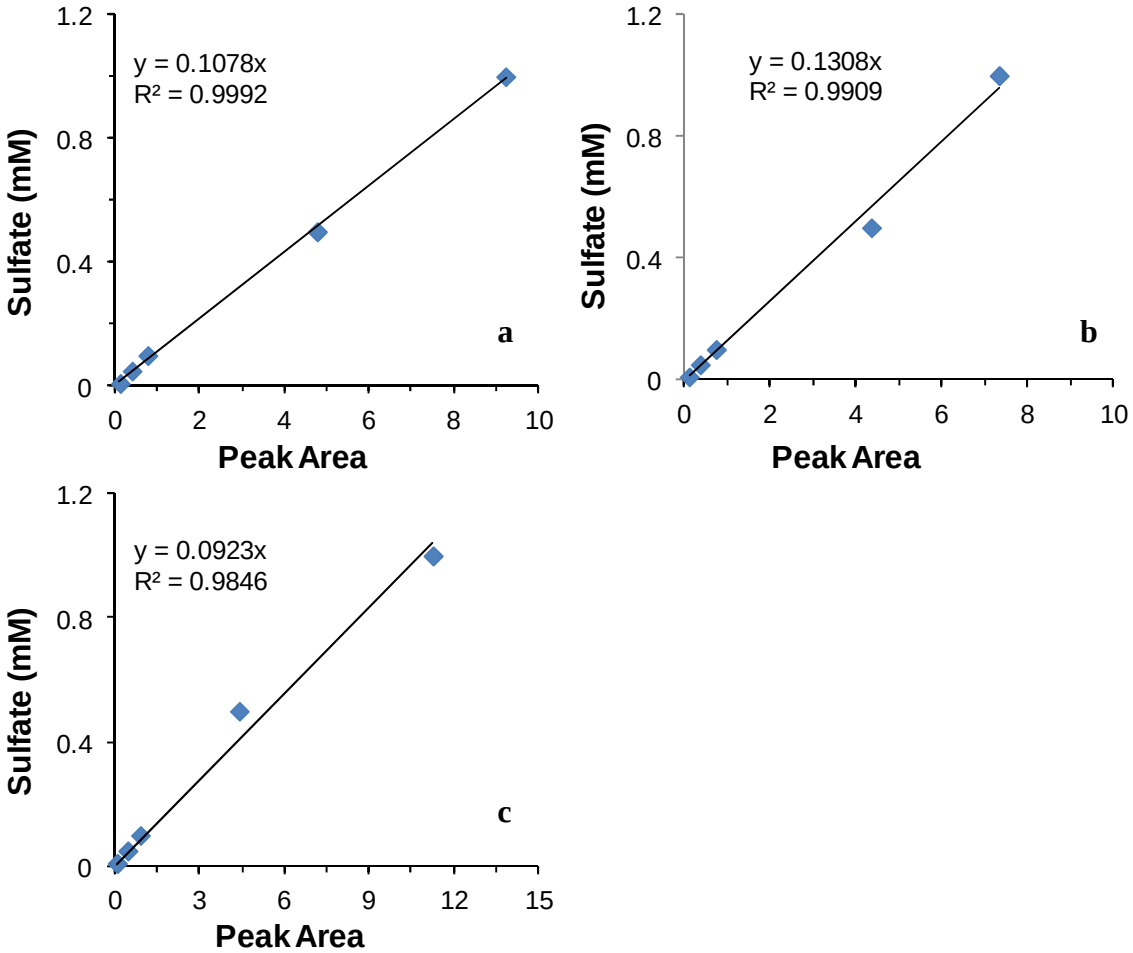

Figure C.1 IC response factor curves for sulfate from 8/18/10 (a), 9/6/10 (b), and 10/24/10 (c).

Appendix D Media Protocol

D.1 Preparation of medium for the DHM-1

Solutions needed for media:

-Trace Metal Solution

In a 1 L volumetric flask, add 1500 mg of $FeCl_2 \cdot 4H_2O$, 190 mg of $CoCl_2 \cdot 6H_2O$, 100 mg of $MnCl_2 \cdot 4H_2O$, 70 mg of $ZnCl_2$, 6 mg of H_3BO_4, 36 mg of $Na_2MoO_4 \cdot 2H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, and 2 mg of $CuCl_2 \cdot 2H_2O$. Then fill to 1 L with DDI water.

-Selenite Solution

In a 1 L volumetric flask, add 3 mg of Na_2SeO_3 and 5 mg of $NaHCO_3$. Then fill to 1 L with DDI water.

-Ferrous Solution

In a 1 L volumetric flask, add 3 mg of $FeCl_2 \cdot 4H_2O$ and 5 mg of $NaHCO_3$. Then fill to 1 L with DDI water.

-Redox Solution

In a 100 mL volumetric flask, add 0.1 g resazurin. Then fill to 100 mL with DDI water.

-Yeast Extract Solution

In a 100 mL volumetric flask, add 5 g yeast extract. Then fill to 100 mL with DDI water.

Media Preparation:
In a 1 L volumetric flask, add 300 mg of NH_4Cl, 300 mg of KCl, 150 mg of $CaCl_2 \cdot 2H_2O$, 400 mg of $MgCl_2 \cdot 6H_2O$, 410 mg of K_2HPO_4, 4 g of $NaHCO_3$, 1 mL of trace metal solution, 1 mL of selenite solution, 1 mL of ferrous solution, 1 mL of redox solution, and 1 mL of sterilized yeast extract solution. Then fill to 1 L with autoclaved DDI water. Transfer to 1 L bottle and purge with 30% CO_2 and 70% N_2 for a few minutes until complete dissolution of mineral salts. Add 120 mg of $Na_2S \cdot 9H_2O$ crystals that have been rinsed and blotted dry with Kimwipes in a completely anaerobic chamber and allow solution to turn from blue to clear.

D.2 Preparation of medium for the SRB culture

Solutions needed for media:

-Trace Metal Solution

In a 1 L volumetric flask, add 1500 mg of $FeCl_2 \cdot 4H_2O$, 190 mg of $CoCl_2 \cdot 6H_2O$, 100 mg of $MnCl_2 \cdot 4H_2O$, 70 mg of $ZnCl_2$, 6 mg of H_3BO_4, 36 mg of $Na_2MoO_4 \cdot 2H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, and 2 mg of $CuCl_2 \cdot 2H_2O$. Then fill to 1 L with DDI water.

-Selenite Solution

In a 1 L volumetric flask, add 3 mg of Na_2SeO_3 and 5 mg of $NaHCO_3$. Then fill to 1 L with DDI water.

-Ferrous Solution

In a 1 L volumetric flask, add 3 mg of $FeCl_2.4H_2O$ and 5 mg of $NaHCO_3$. Then fill to 1 L with DDI water.

-Redox Solution

In a 100 mL volumetric flask, add 0.1 g resazurin. Then fill to 100 mL with DDI water.

-Yeast Extract Solution

In a 100 mL volumetric flask, add 5 g yeast extract. Then fill to 100 mL with DDI water.

Media Preparation:

In a 1 L volumetric flask, add 300 mg of NH_4Cl, 300 mg of KCl, 150 mg of $CaCl_2 \cdot 2H_2O$, 400 mg of $MgCl_2 \cdot 6H_2O$, 410 mg of K_2HPO_4, 4 g of $NaHCO_3$, 5.268 g of Na_2SO_4, 1 mL of trace metal solution, 1 mL of selenite solution, 1 mL of ferrous solution, 1 mL of redox solution, and 1 mL of sterilized yeast extract solution. Then fill to 1 L with autoclaved DDI water. Transfer to 1 L bottle. Add 120 mg of $Na_2S \cdot 9H_2O$ crystals that have been rinsed and blotted dry with Kimwipes in a completely anaerobic chamber and allow solution to turn from blue to clear.

D.3 Preparation of medium for the DCM culture

Solutions needed for media:

- Phosphate buffer

In a 100 mL volumetric flask add 5.25 g K_2HPO_4. Then fill to 100 mL with DDI water.

- Salt solution

In a 100 mL volumetric flask add 5.35 g NH_4Cl, 0.46976 g $CaCl_2$:$2H_2O$, and 0.17787 g $FeCl_2$:H_2O. Then fill to 100 mL with DDI water.

- Trace metals solution

In a 100 mL volumetric flask add 0.03 g H_3BO_3, 0.0211 g $ZnSO_4$:$7H_2O$, 0.075 g $NiCl_2$:6H2O, 0.1 g $MnCl_2$:$4H_2O$, 0.01 g $CuCl_2$:$2H_2O$, 0.15 g $CoCl_2$:$6H_2O$, 0.002 g Na_2SeO_3, 0.01 g $Al_2(SO_4)_3$:$16H_2O$, and 1 mL concentrated HCl. Then fill to 100 mL with DDI water.

- Magnesium sulfate solution

In a 100 mL volumetric flask add 6.25 g $MgSO_4$:$7H_2O$. Then fill to 100 mL with DDI water.

- Bicarbonate solution

In a 500 mL volumetric flask add 8.0 g $NaHCO_3$. Then fill to 500 mL with DDI water.

- Redox solution

In a 10 mL volumetric flask add 0.01 g resazurin. Then fill to 10 mL with DDI water.

- Ferrous sulfide solution

To be added directly to autoclaved media after adding filter-sterilized solutions and placing in glove box. For 1 L, weigh out 0.24g of $Na_2S:9H_2O$ and 0.1448g $FeCl_2:H_2O$ based on stock concentrations of 24 g/L and 14.48 g/L, into separate vials. Add the $Na_2S:9H_2O$ and allow media to clear. Add the $FeCl_2:H_2O$ and rinse both vials out with the 10 mL of autoclaved DDI water needed to balance the solution and add to the media.

-Yeast extract solution

In a 100 mL volumetric flask add 0.5 g yeast extract. Then fill to 100 mL with DDI water.

Media Preparation:

In a 1 L bottle add 10 mL phosphate solution, 10 mL salt solution, 2 mL trace metals solution, 2 mL magnesium sulfate solution, 1 mL redox solution, and 905 mL DDI water. Autoclave this solution, and then add 50 mL filter sterilized bicarbonate solution and 10 mL filter sterilized yeast extract. In the glove box, add the 10 mL ferrous sulfide solution.

Appendix E Figures for Individual Microcosm Bottles from Site A

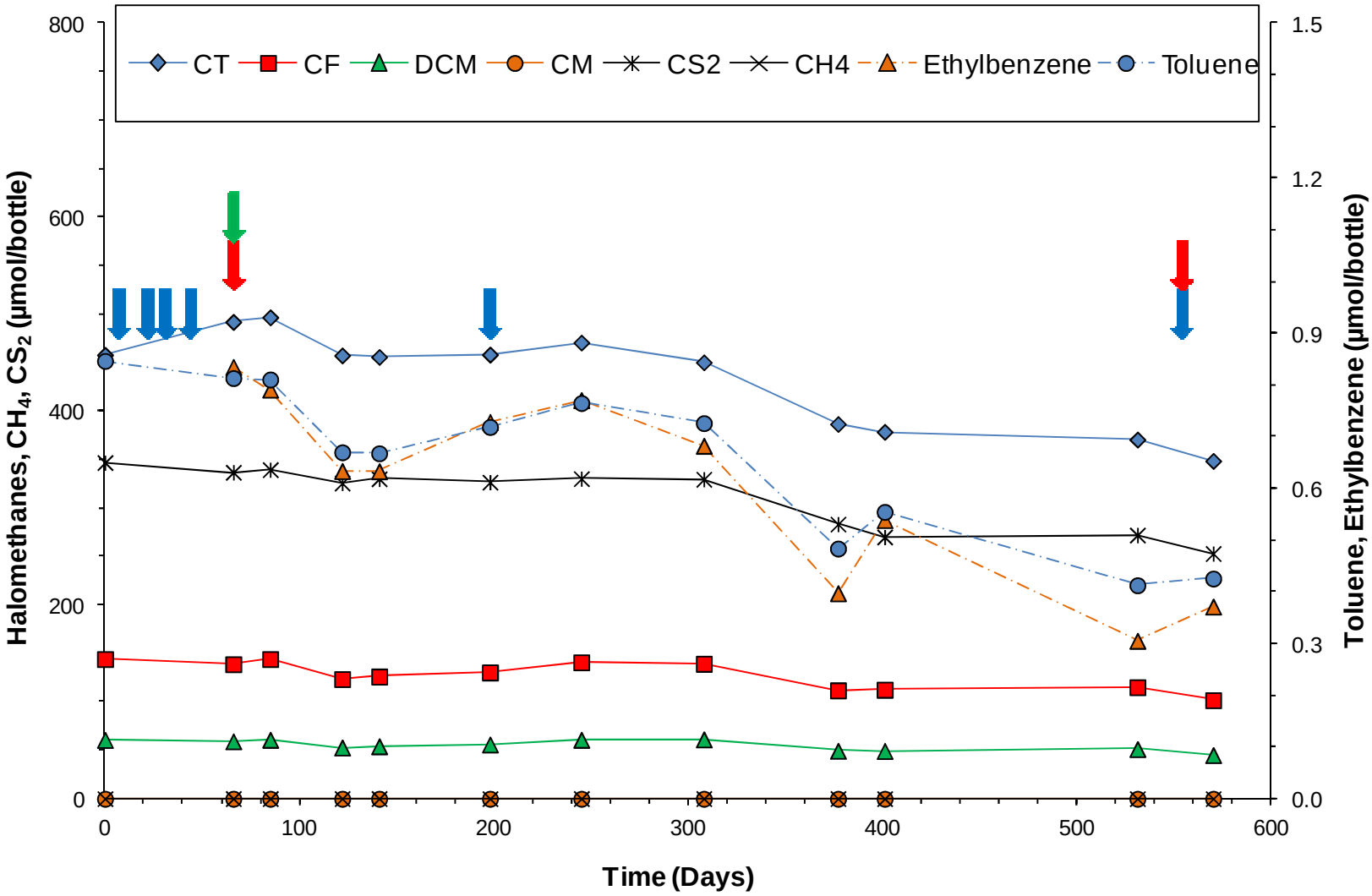

Figure E.1 Results for Site A, high concentration plume, bioaugmentation treatment A (bottle #1); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of DHM-1.

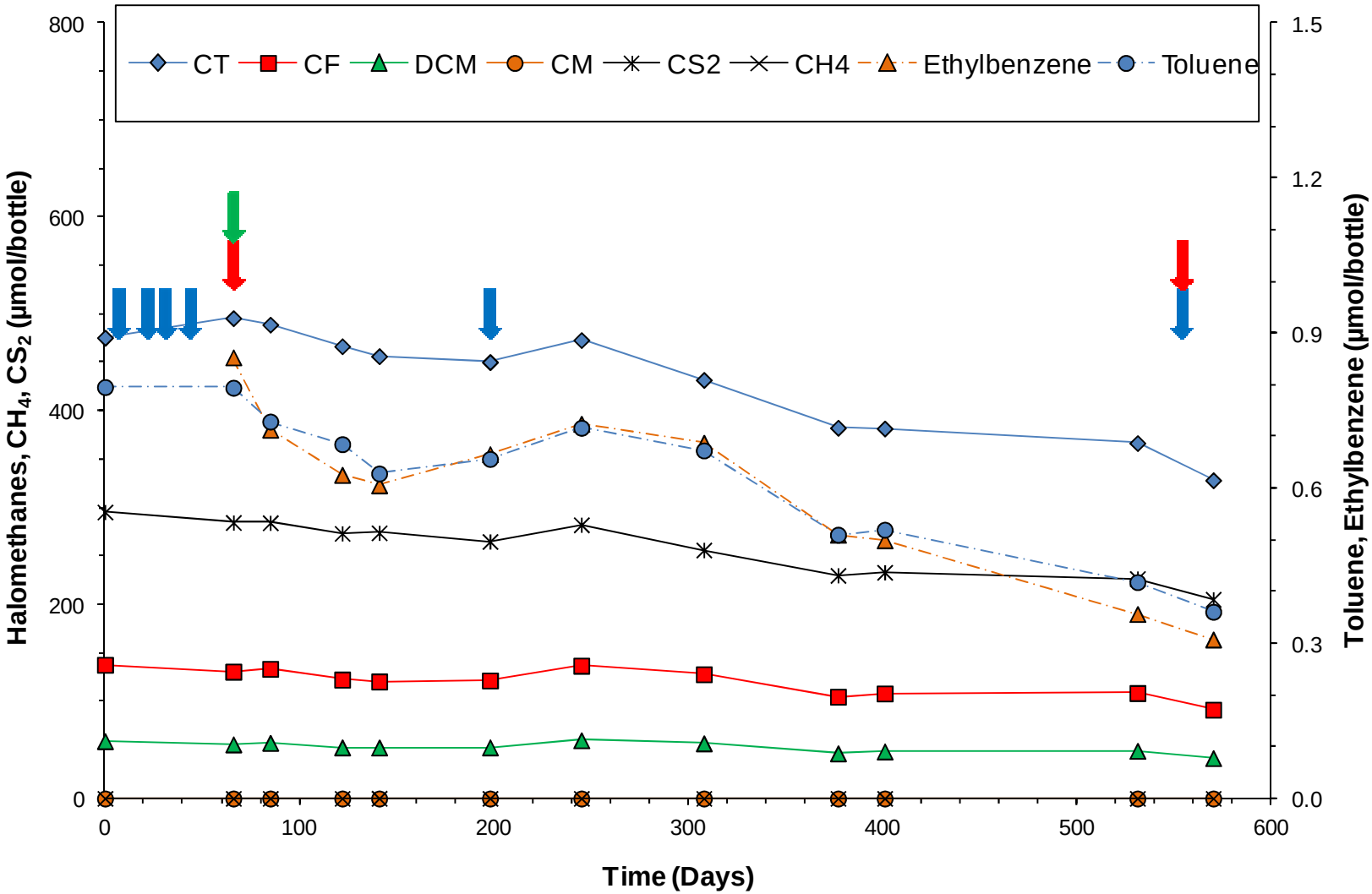

Figure E.2 Results for Site A, high concentration plume, bioaugmentation treatment A (bottle #2); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of DHM-1.

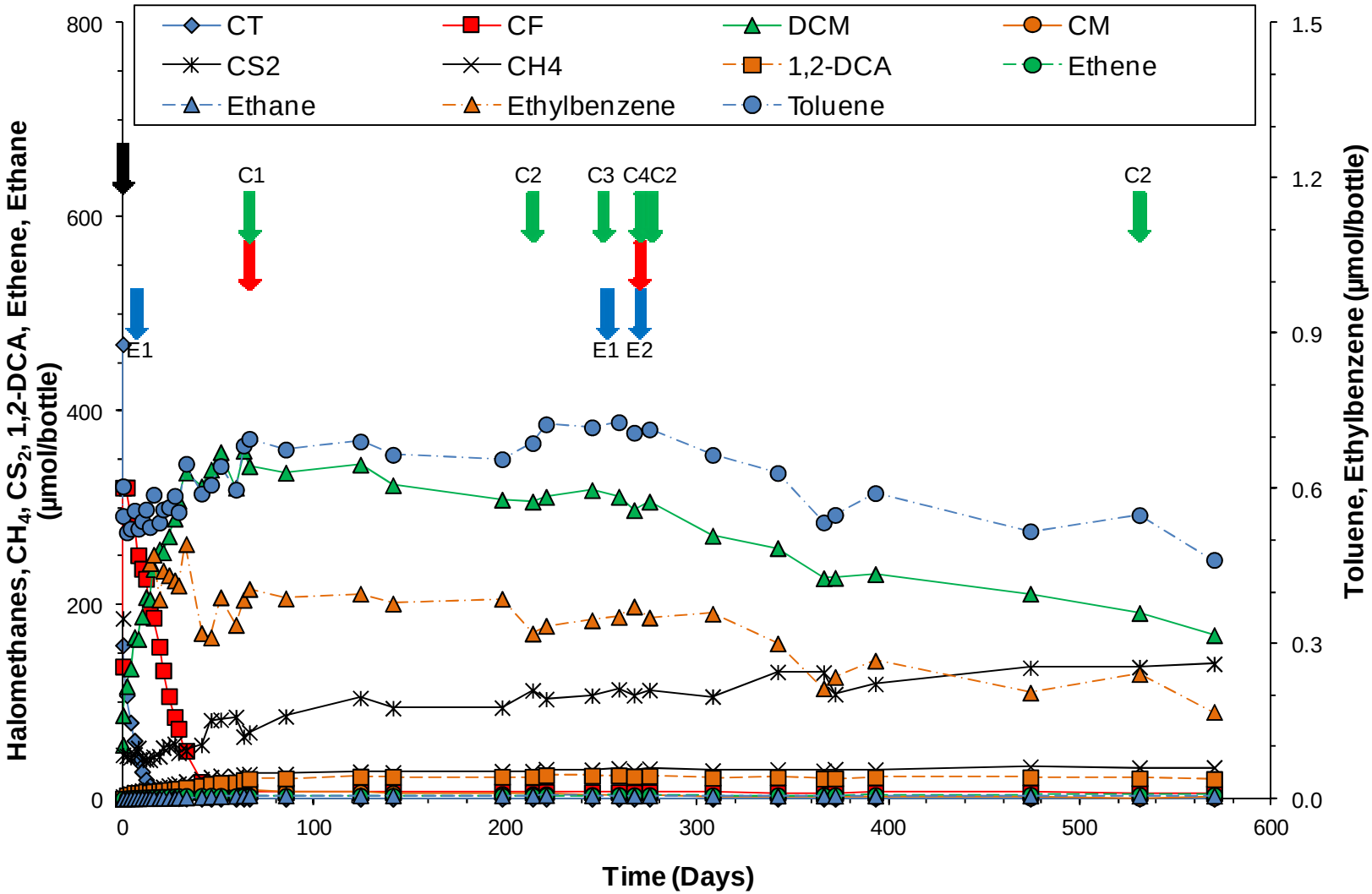

Figure E.3 Results for Site A, high concentration plume, ZVI + bioaugmentation treatment (bottle #2); ⬇= addition of ZVI; ⬇= addition of electron donor (E1 = lactate, E2 = corn syrup); ⬇ = addition of B_{12}; ⬇ = addition of cultures (C1 = SDC-9, C2 = DCM, C3 = 1,2-DCA respiring culture, and C4 = DHM-1).

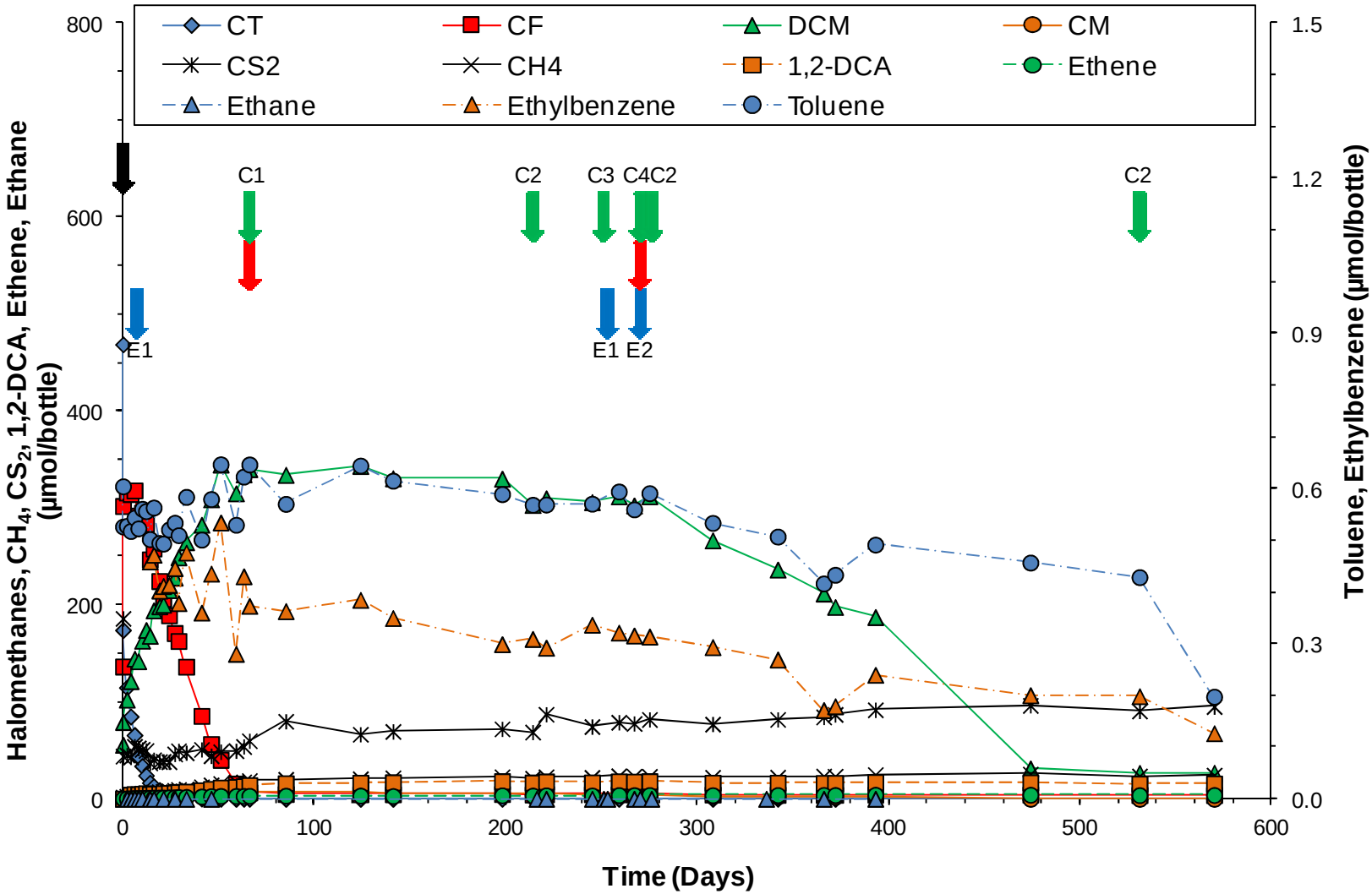

Figure E.4 Results for Site A, high concentration plume, ZVI + bioaugmentation treatment (bottle #3); ⬇= addition of ZVI; ⬇= addition of electron donor (E1 = lactate, E2 = corn syrup); ⬇= addition of B_{12}; ⬇= addition of cultures (C1 = SDC-9, C2 = DCM, C3 = 1,2-DCA respiring culture, and C4 = DHM-1).

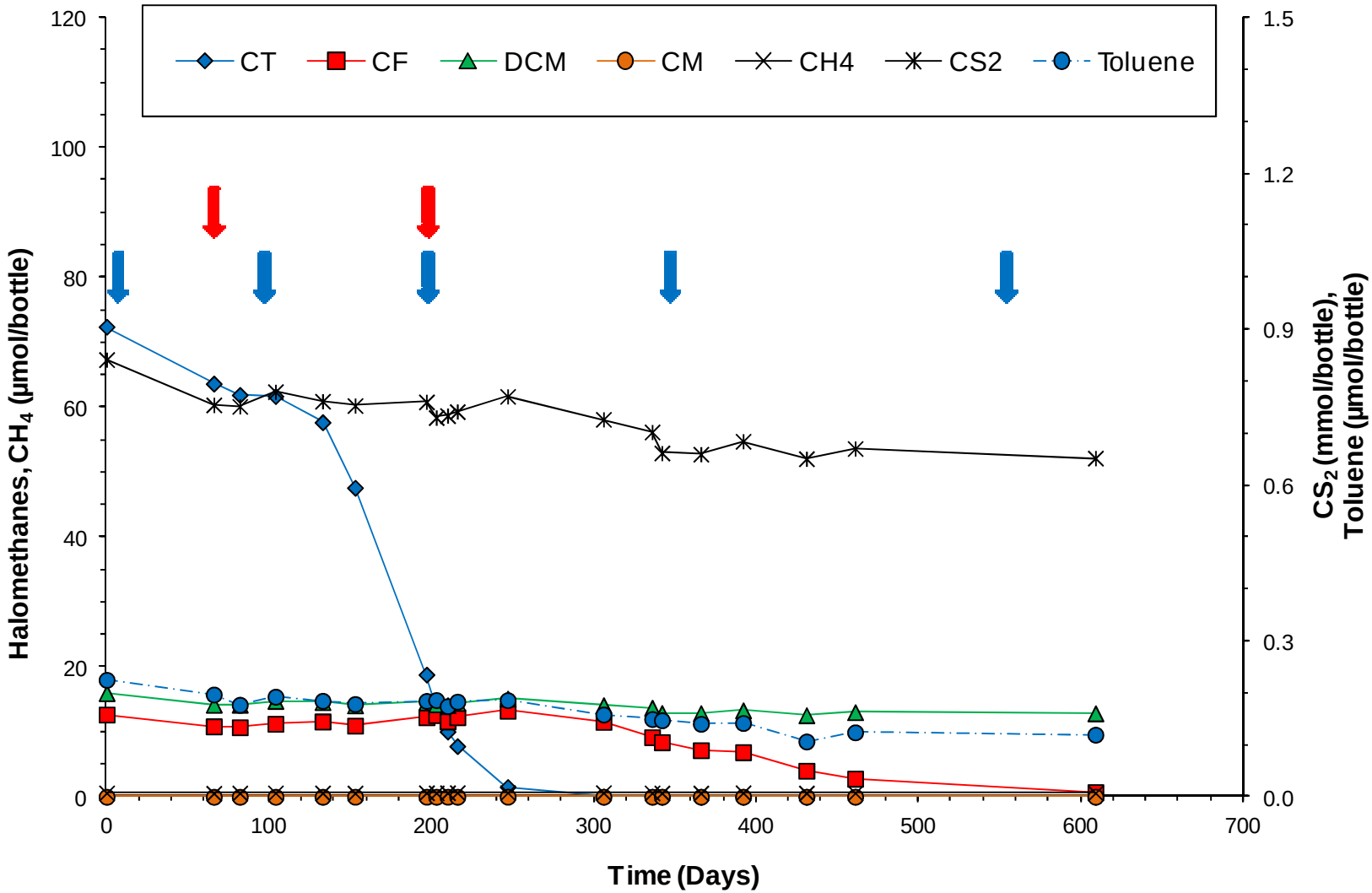

Figure E.5 Results for Site A, medium concentration plume, biostimulation with corn syrup + B_{12} (bottle #1); ⬇ = addition of corn syrup; ⬇ = addition of B_{12}.

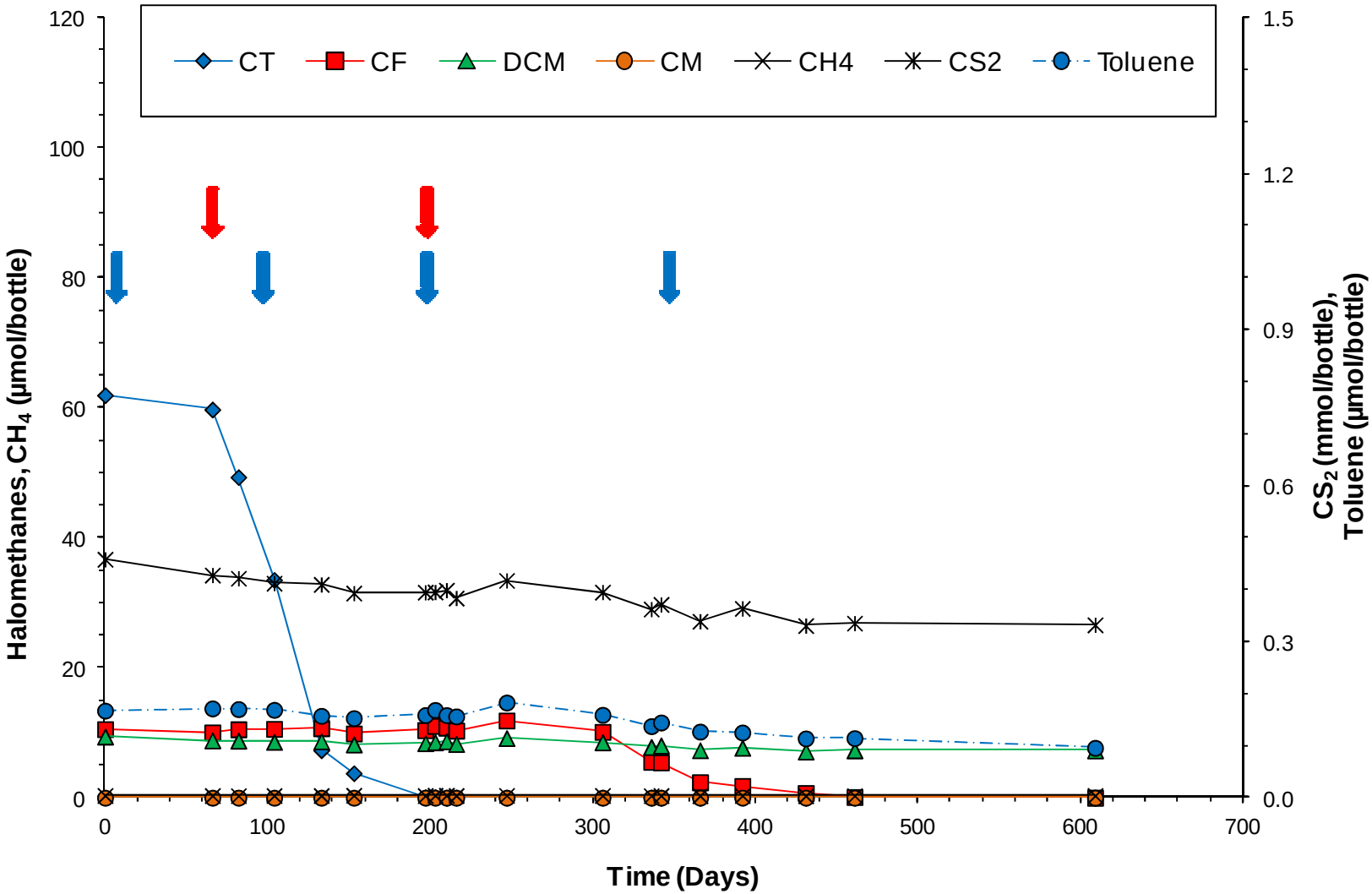

Figure E.6 Results for Site A, medium concentration plume, biostimulation with corn syrup + B_{12} (bottle #3); ⬇ = addition of corn syrup; ⬇ = addition of B_{12}.

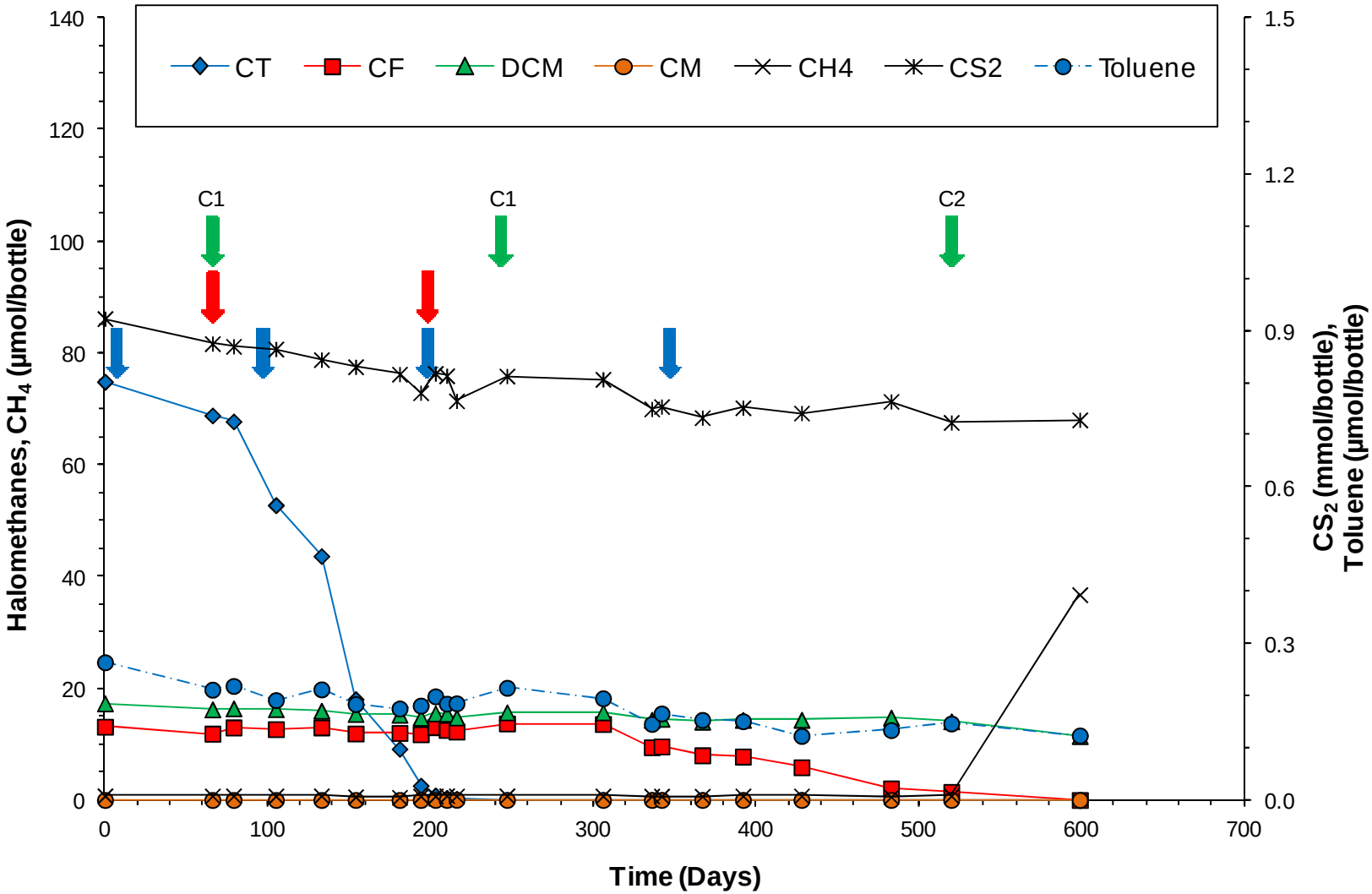

Figure E.7 Results for Site A, medium concentration plume, bioaugmentation treatment A (bottle #1); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of cultures (C1 = DHM-1, C2 = DCM).

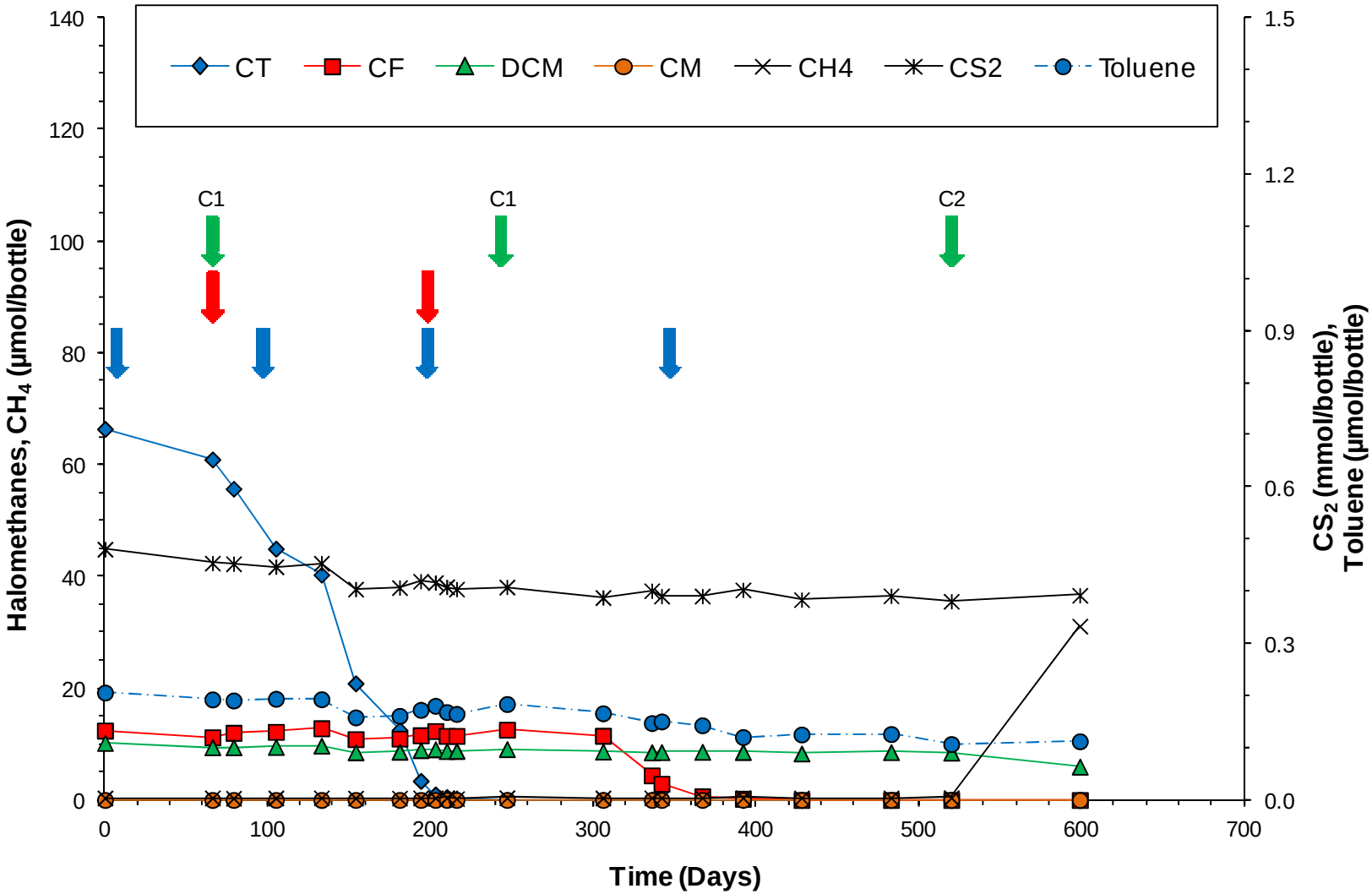

Figure E.8 Results for Site A, medium concentration plume, bioaugmentation treatment A (bottle #3); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of cultures (C1 = DHM-1, C2 = DCM).

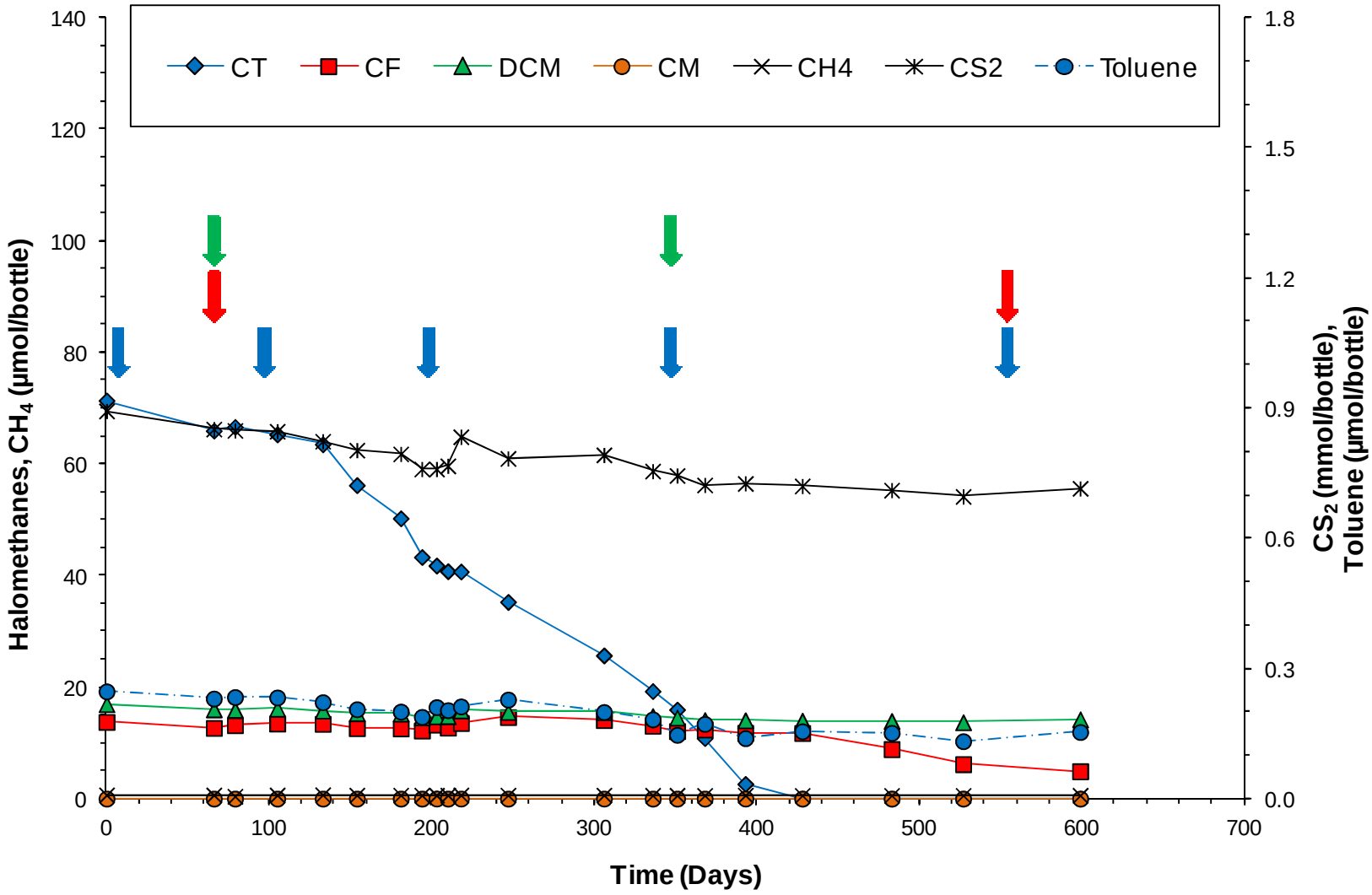

Figure E.9 Results for Site A, medium concentration plume, bioaugmentation treatment B (bottle #1); ↓ = addition of lactate; ↓ = addition of B_{12}; ↓ = addition of SDC-9.

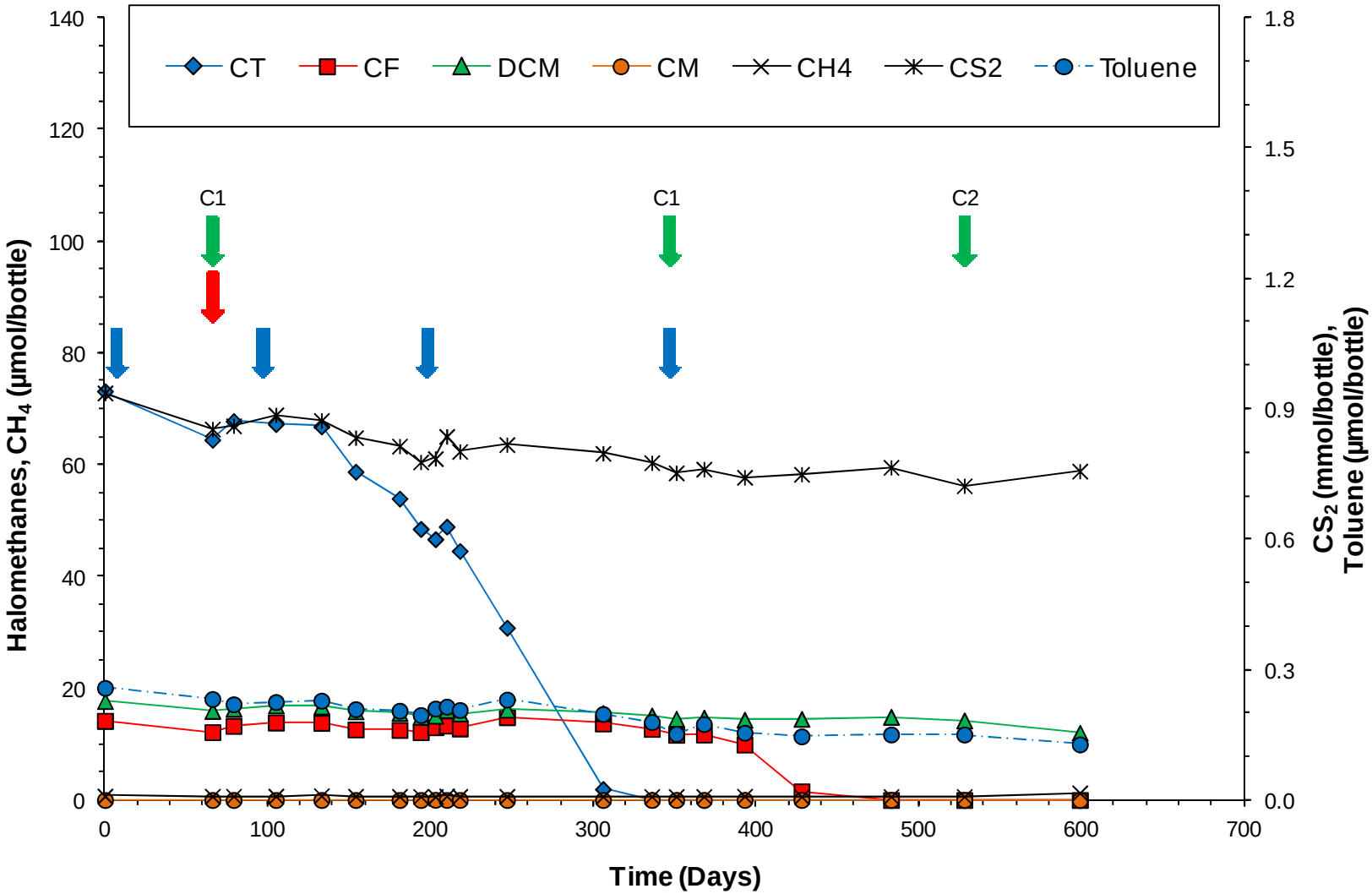

Figure E.10 Results for Site A, medium concentration plume, bioaugmentation treatment B (bottle #3); ↓ = addition of lactate; ↓ = addition of B_{12}; ↓ = addition of cultures (C1 = SDC-9, C2 = DCM).

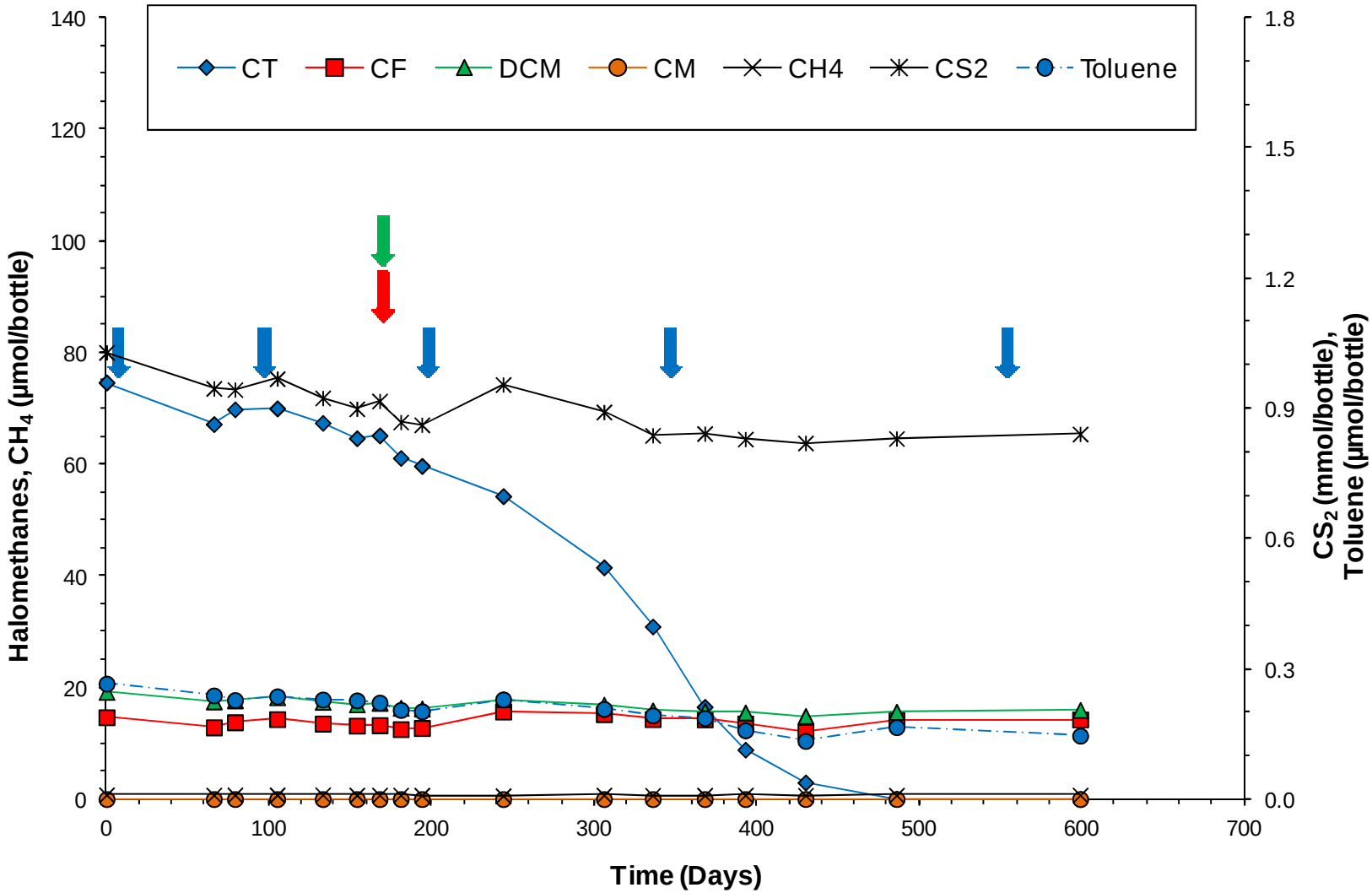

Figure E.11 Results for Site A, medium concentration plume, bioaugmentation treatment C (bottle #2); ⬇ = addition of lactate; ⬇ = addition of B_{12}; ⬇ = addition of SRB.

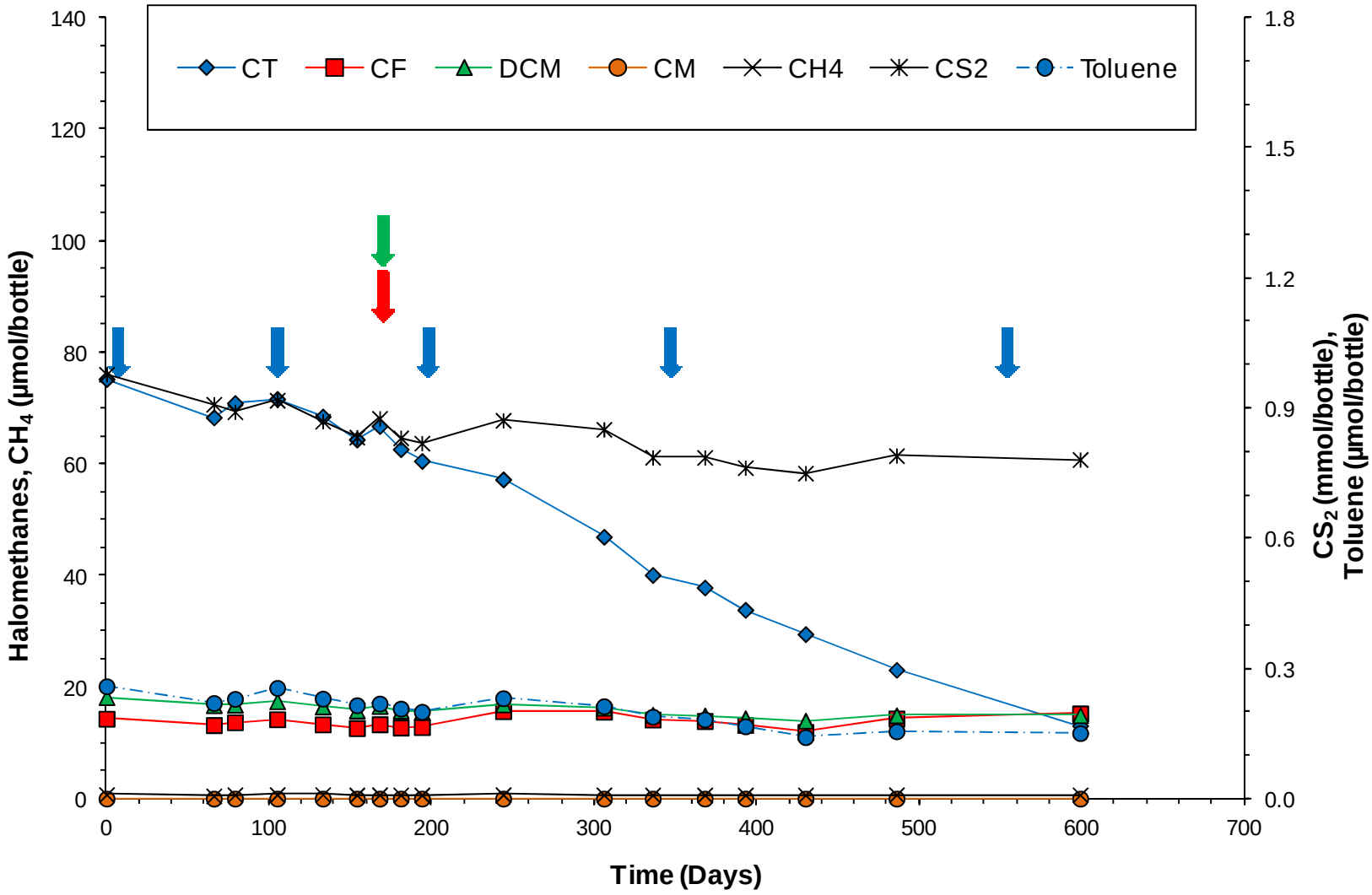

Figure E.12 Results for Site A, medium concentration plume, bioaugmentation treatment C (bottle #3); ⬇ = addition of lactate; ⬇ = addition of B_{12}; ⬇ = addition of SRB.

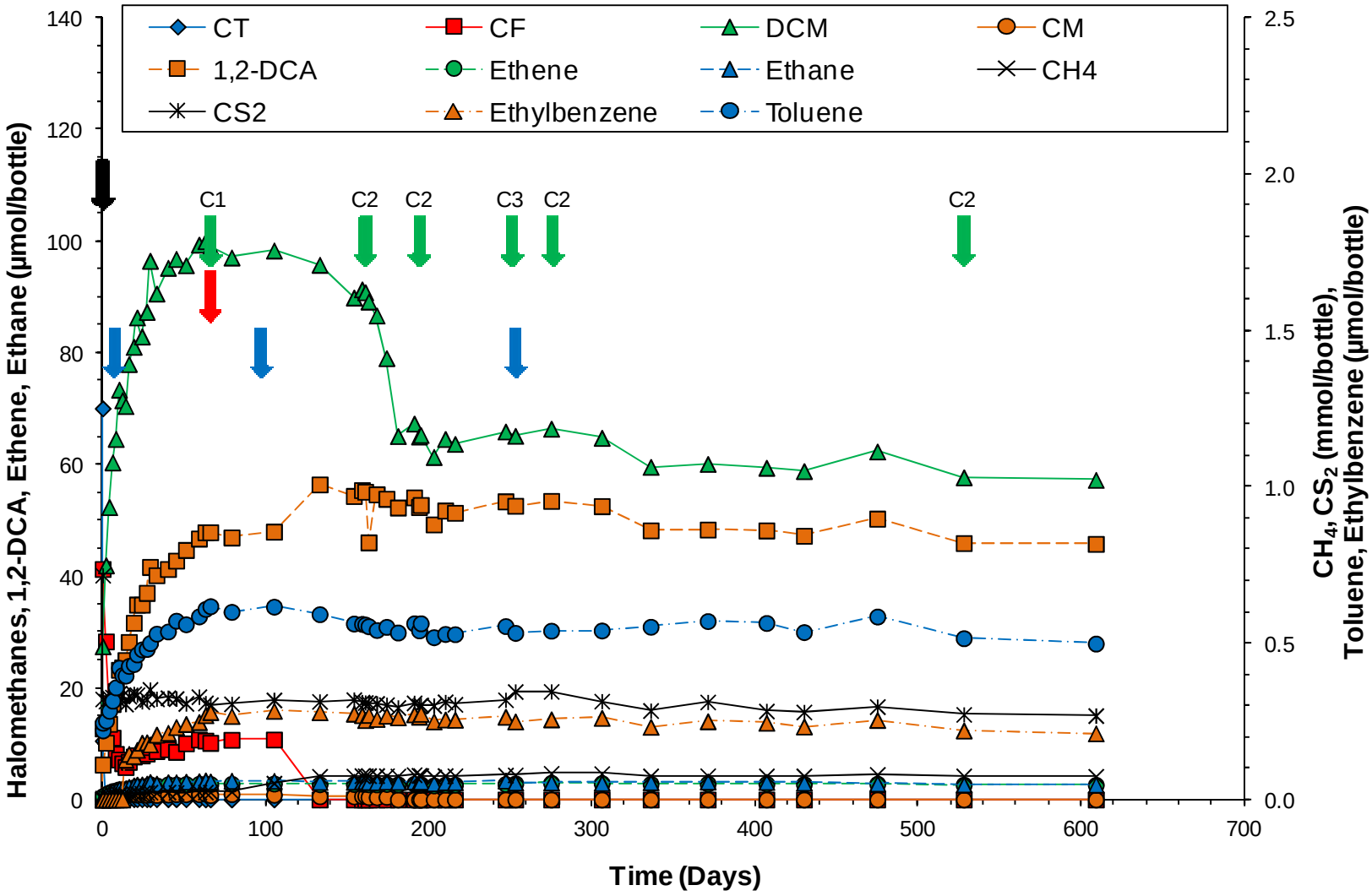

Figure E.13 Results for Site A, medium concentration plume, ZVI + bioaugmentation treatment (bottle #1); ⬇= addition of ZVI; ⬇= addition of lactate; ⬇= addition of B_{12}; ⬇= addition of cultures (C1 = SDC-9, C2 = DCM, and C3 = DCA respiring culture).

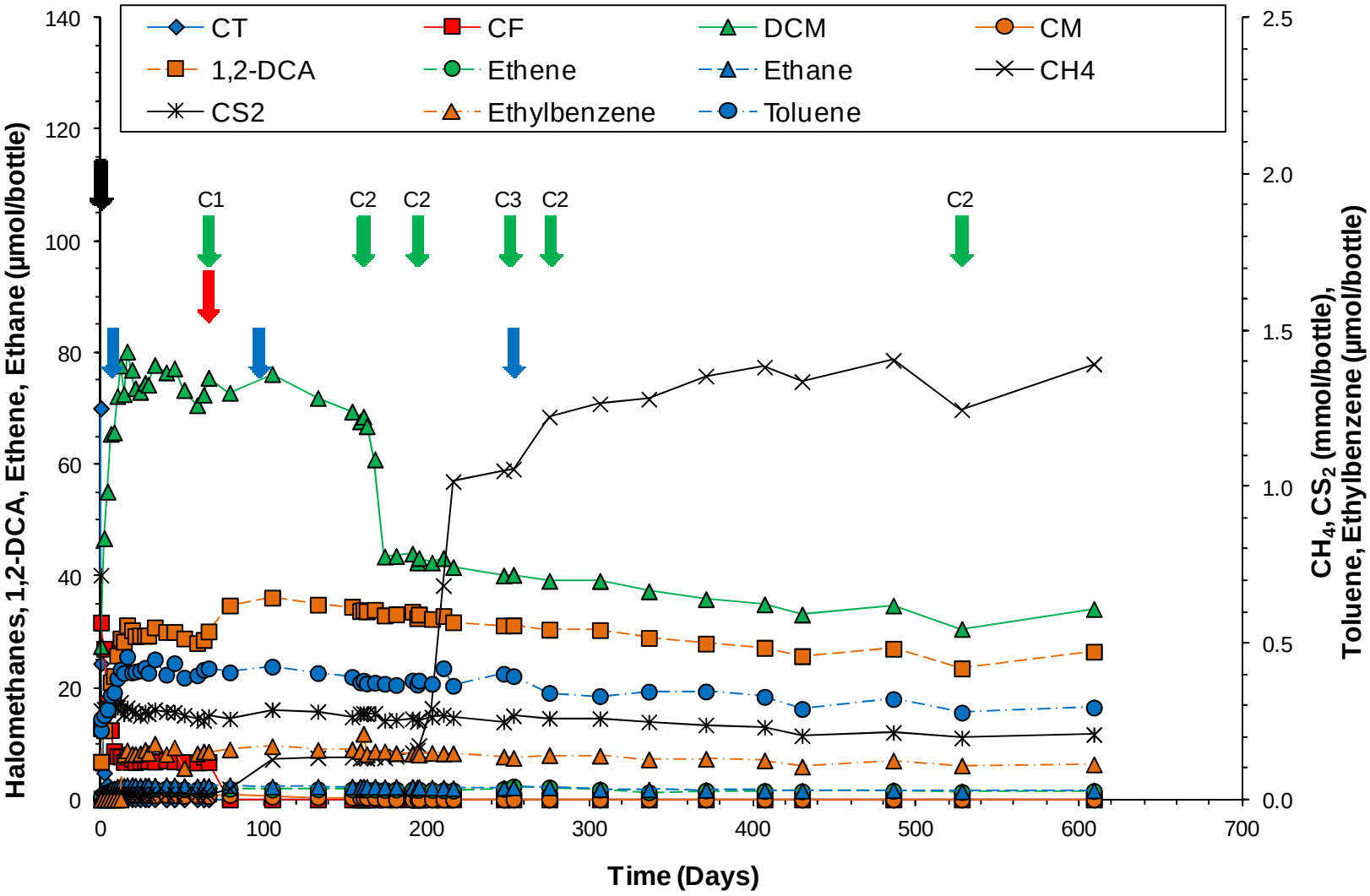

Figure E.14 Results for Site A, medium concentration plume, ZVI + bioaugmentation treatment (bottle #3); ⬇ = addition of ZVI; ⬇ = addition of lactate; ⬇ = addition of B_{12}; ⬇ = addition of cultures (C1 = SDC-9, C2 = DCM, and C3 = DCA respiring culture).

Appendix F Sample Chromatograms for Site A

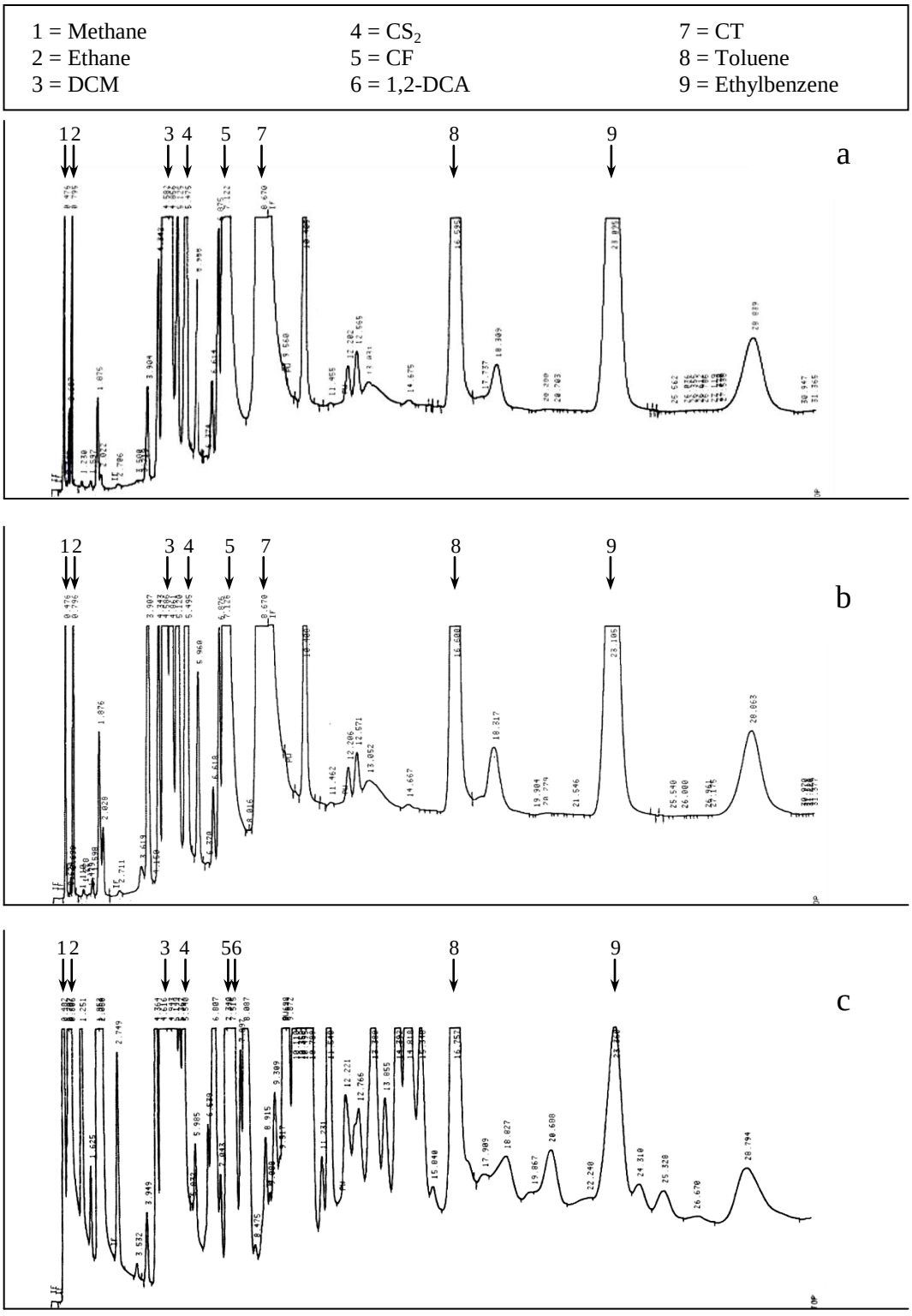

Figure F.1 Sample chromatograms for treatments H-CON (a), H-BIOA (b), and H-ZBIO (c).

Appendix G Groundwater Withdrawal Impacts on GC Response Factors for Site B

In the description of results for Site B (Section 4.2), it was mentioned that repeated withdrawals of groundwater (to measure sulfate) made it necessary to add groundwater in order to restore the balance between the liquid volume and headspace volume. The impact of decreasing the liquid volume on the GC response factors for the primary volatile organic compounds was calculated, based on the distribution of mass between the headspace and liquid phases and the subsequent change in the gas phase concentration. Table G.1 shows the percent difference in gas phase concentration between the bottle at the correct headspace and liquid volumes and the bottle after the maximum amount of groundwater was withdrawn. There was less than a 1% impact on CT, while the maximum impact on CF was 12.3%.

Table G.1 Summary of percent changes on the GC response factors of CT, CF, DCM, 1,2-DCA, and 1,1,2-TCA due to groundwater withdrawals for Site B.

Microcosm Bottle	GW Withdrawal (mL)	GC Response Factor Percent Change				
		CT	CF	DCM	1,2-DCA	1,1,2-TCA
AC-1	8	0.6%	12.3%	14.5%	15.8%	16.9%
AC-2	8	0.6%	12.3%	14.5%	15.8%	16.9%
AC-3	8	0.6%	12.3%	14.5%	15.8%	16.9%
CON-1	7	0.5%	10.6%	12.5%	13.6%	14.5%
CON-2	7	0.5%	10.6%	12.5%	13.6%	14.5%
CON-3	6	0.4%	8.9%	10.5%	11.4%	12.2%
BST-1	8	0.6%	12.3%	14.5%	15.8%	16.9%
BST-2	8	0.6%	12.3%	14.5%	15.8%	16.9%
BST-3	8	0.6%	12.3%	14.5%	15.8%	16.9%
BB12-1	6	0.4%	8.9%	10.5%	11.4%	12.2%
BB12-2	7	0.5%	10.6%	12.5%	13.6%	14.5%
BB12-3	7	0.5%	10.6%	12.5%	13.6%	14.5%
BIO-1	6	0.4%	8.9%	10.5%	11.4%	12.2%
BIO-2	6	0.4%	8.9%	10.5%	11.4%	12.2%
BIO-3	6	0.4%	8.9%	10.5%	11.4%	12.2%

Appendix H Figures for Individual Bottles from B_{12} Test on the DHM-1 Culture

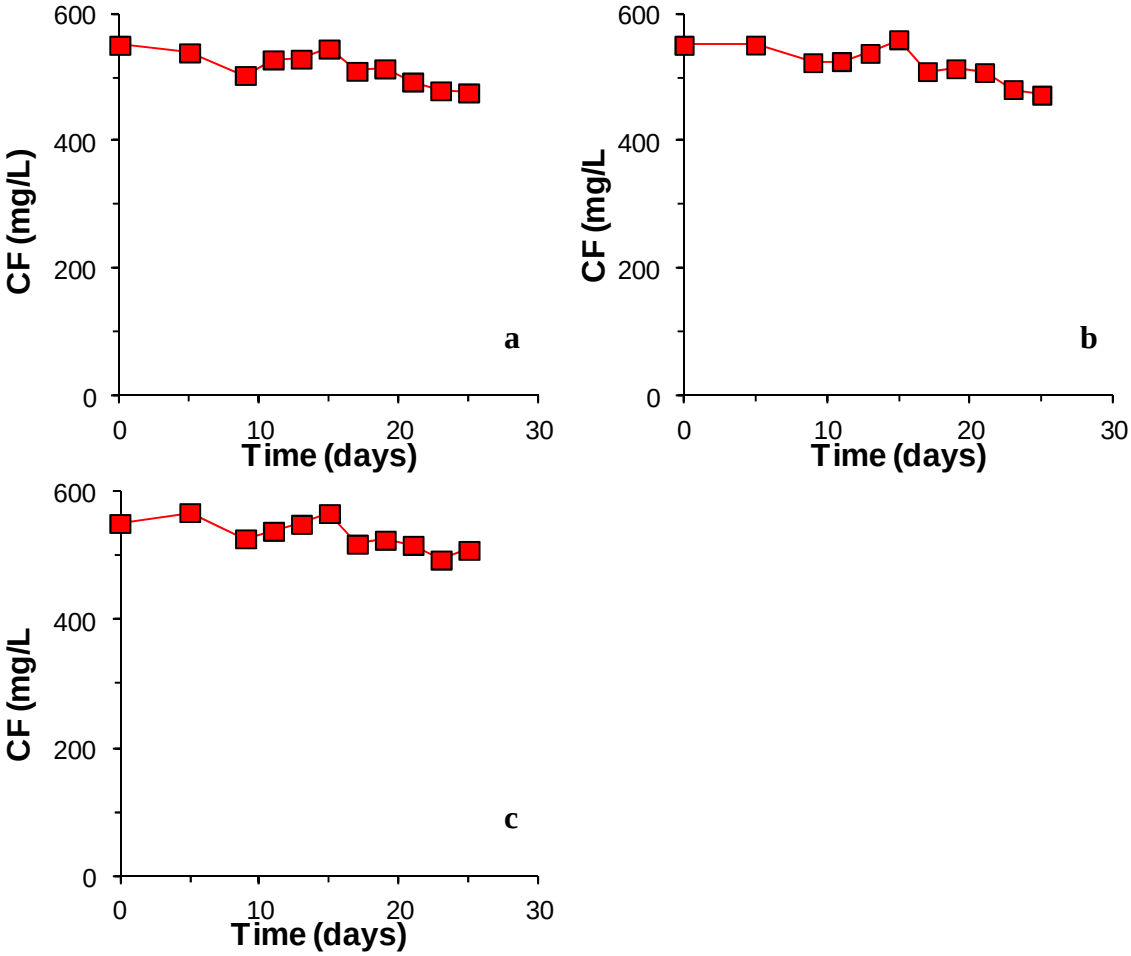

Figure H.1 CF transformation by DHM-1 when B_{12} concentration is 0% in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

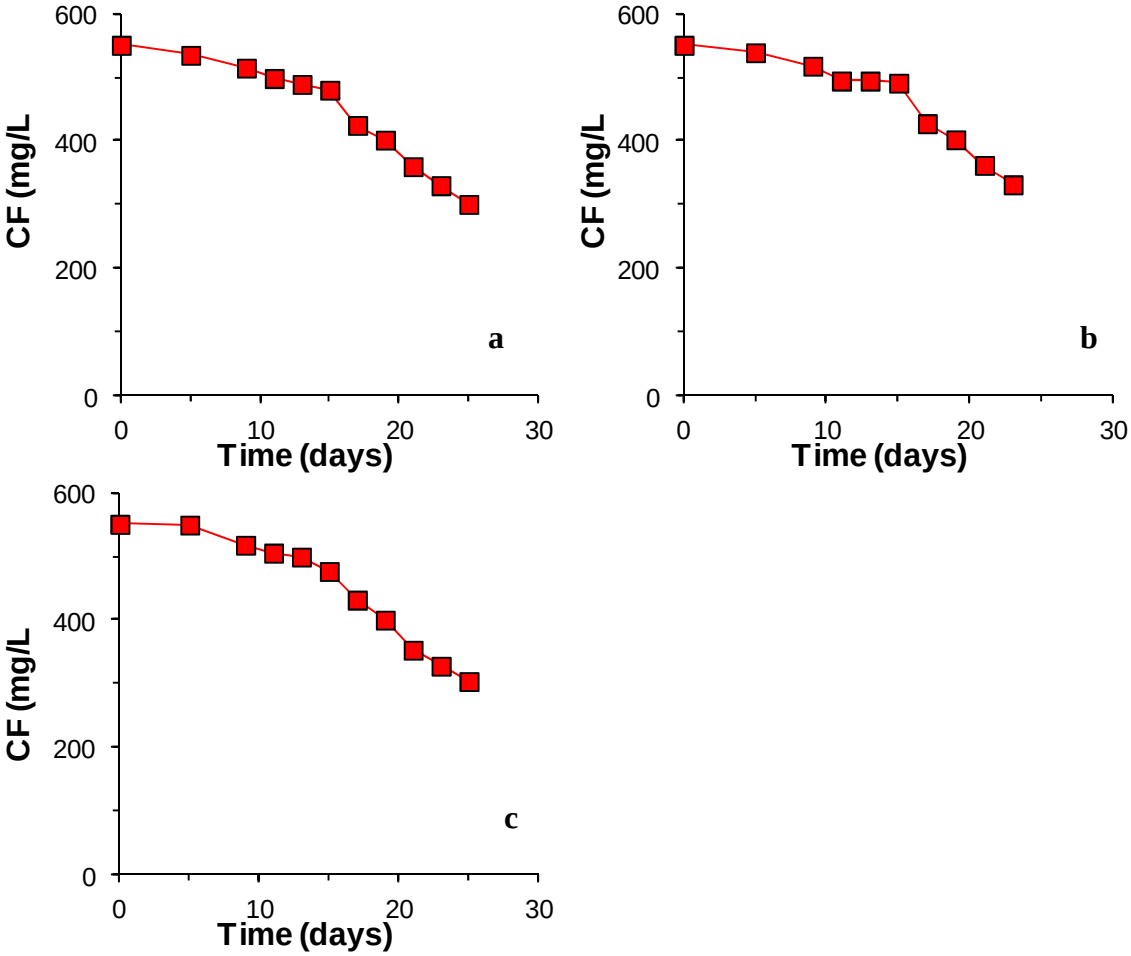

Figure H.2 CF transformation by DHM-1 when B_{12} concentration is 0.15% in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

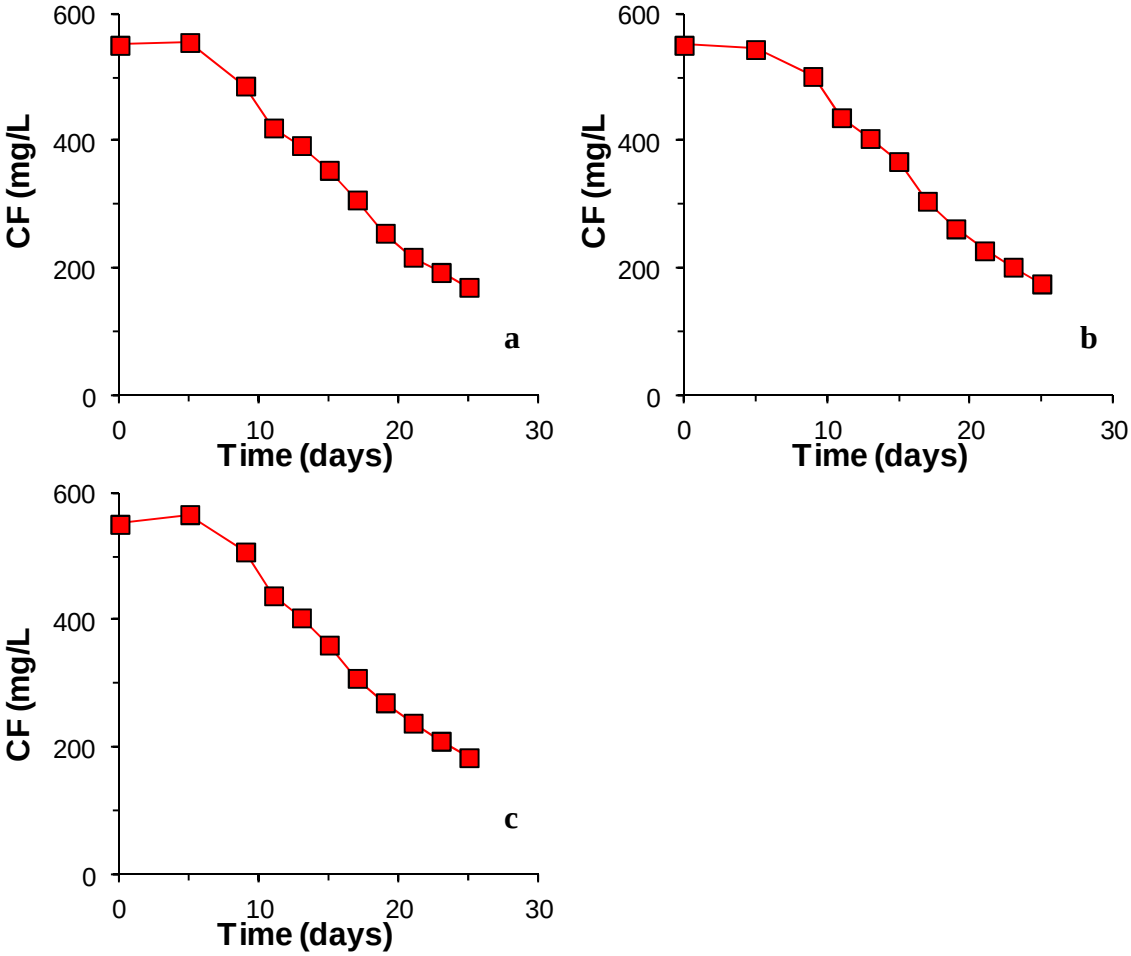

Figure H.3 CF transformation by DHM-1 when B_{12} concentration is 0.3% in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

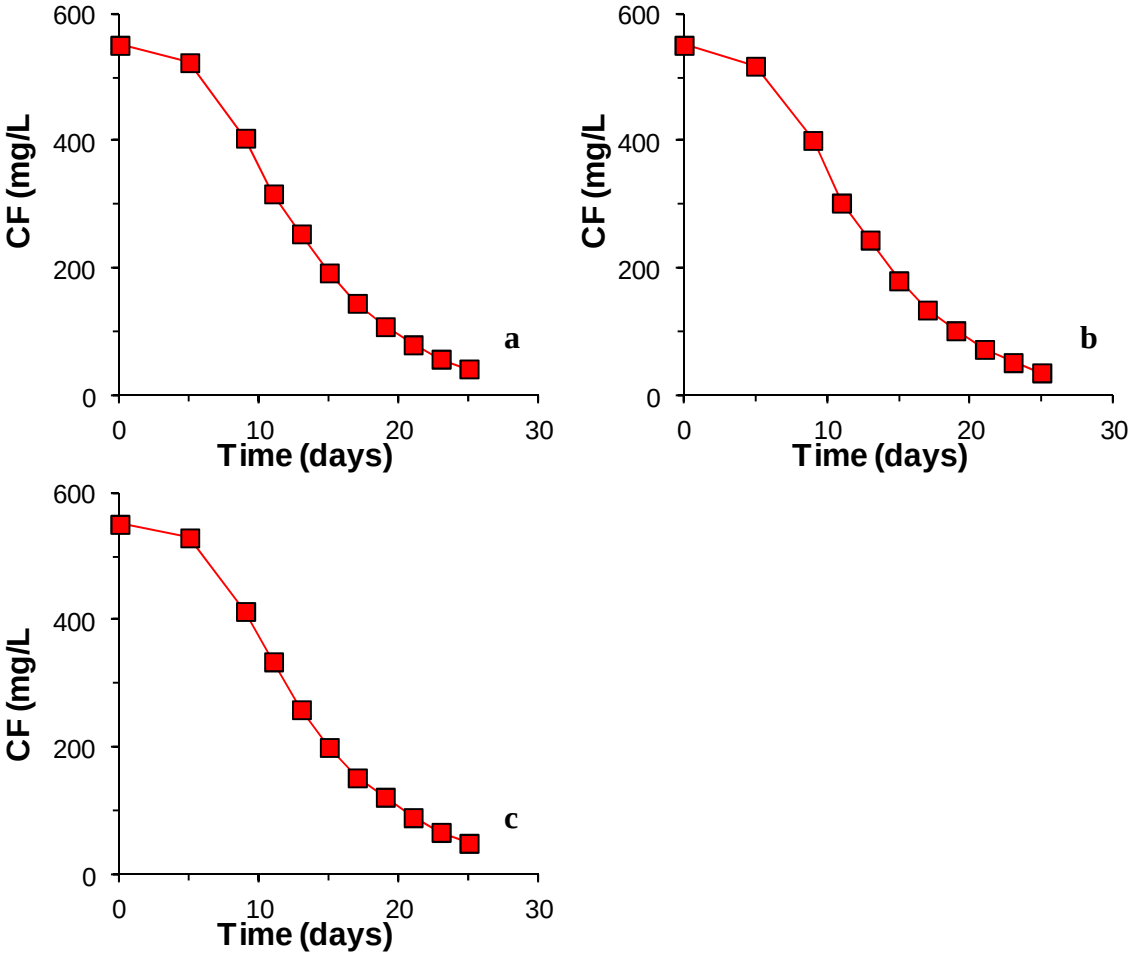

Figure H.4 CF transformation by DHM-1 when B_{12} concentration is 0.6% in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

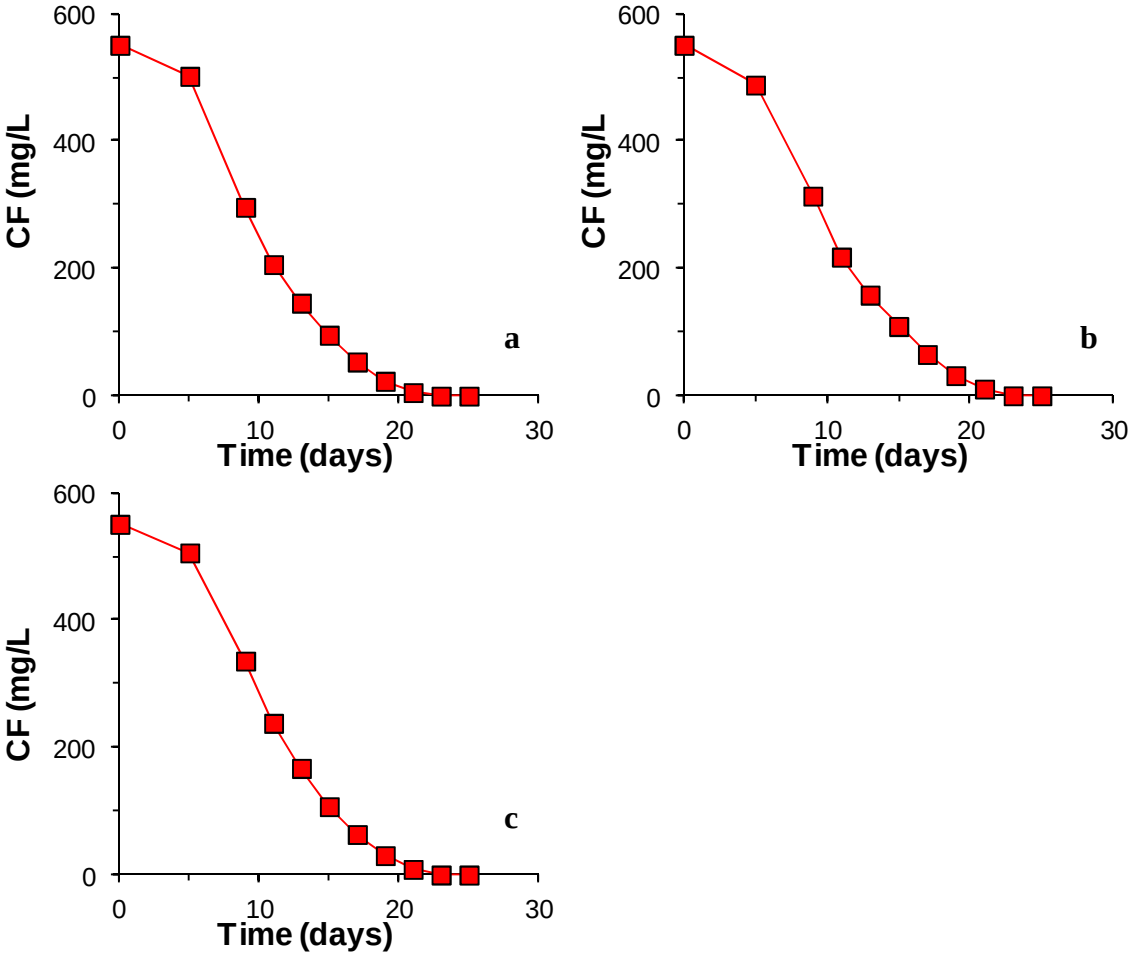

Figure H.5 CF transformation by DHM-1 when B_{12} concentration is 1% in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

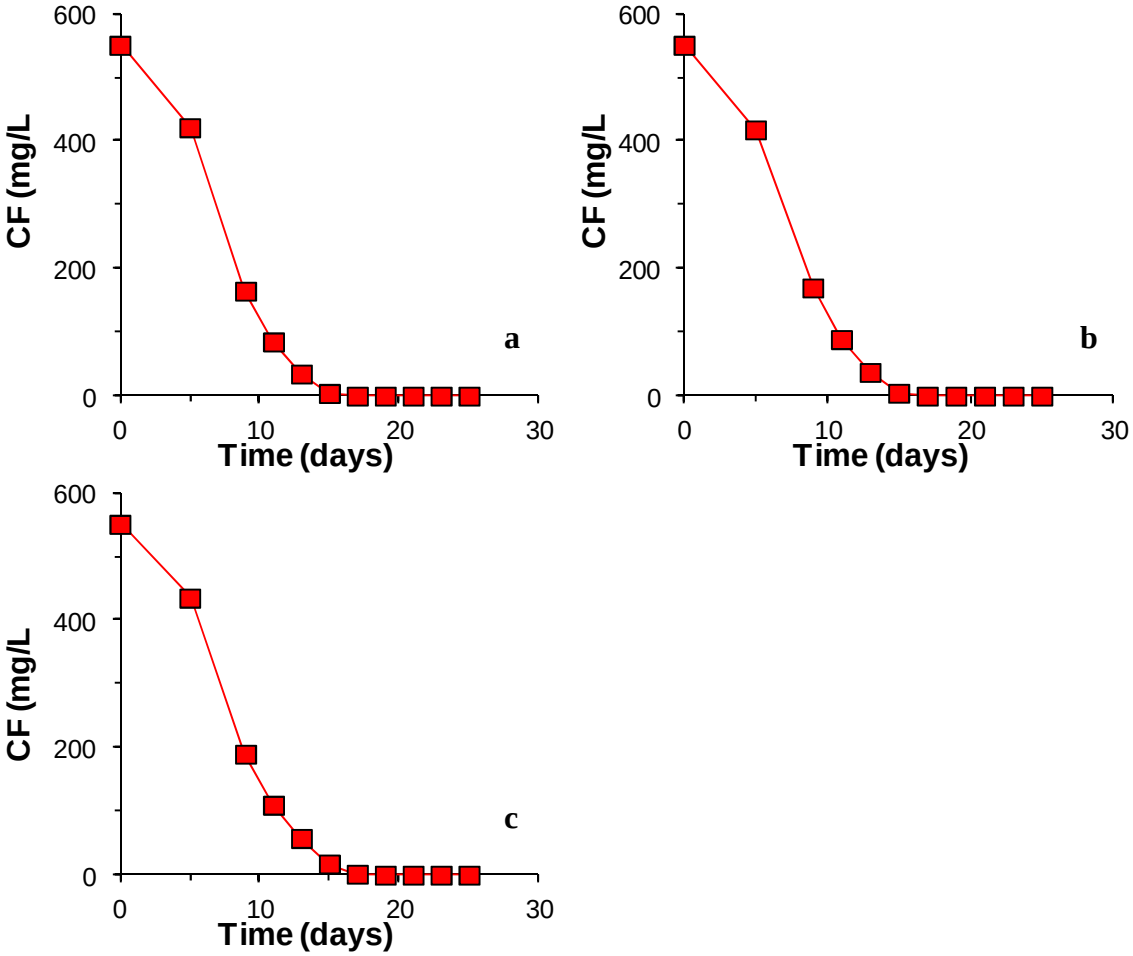

Figure H.6 CF transformation by DHM-1 when B_{12} concentration is 3% in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

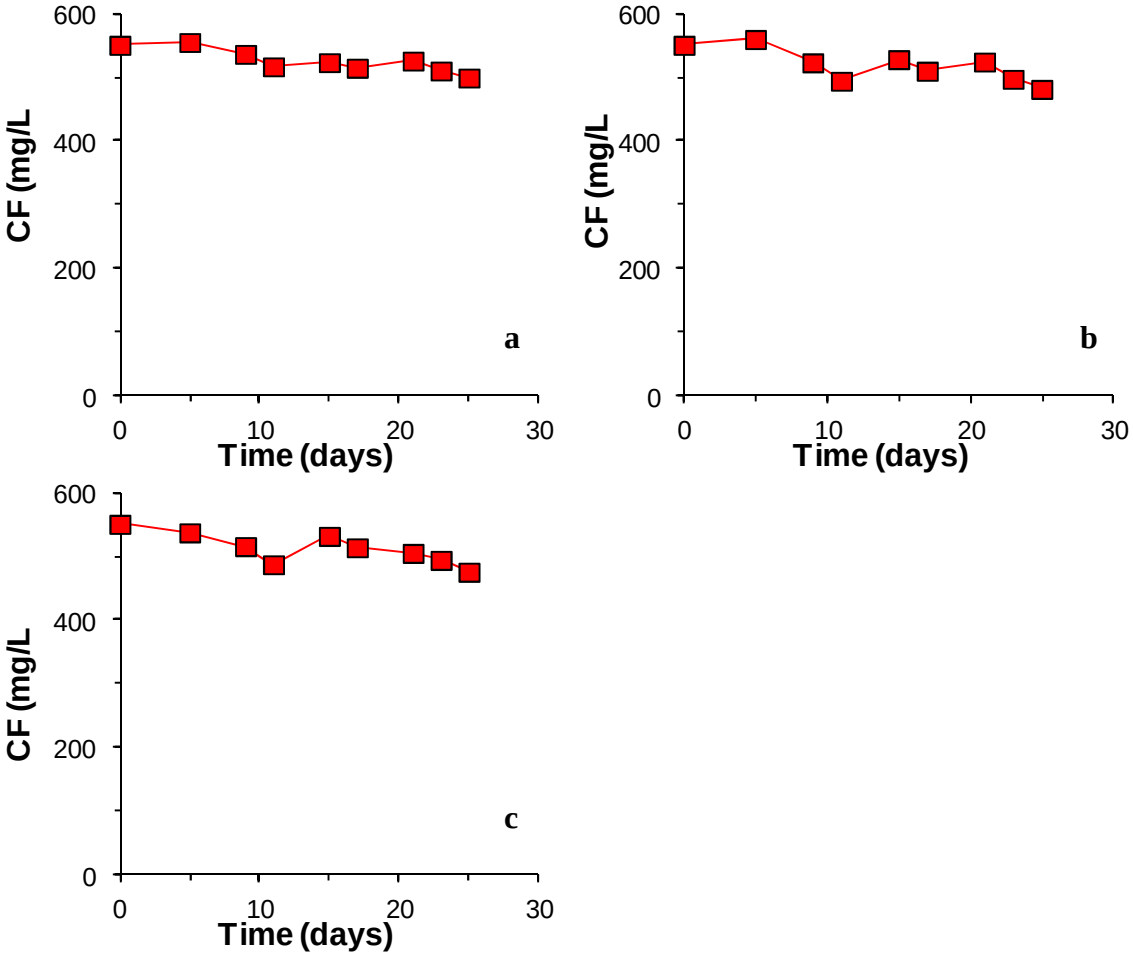

Figure H.7 CF transformation in medium control bottle 1 (a), bottle 2 (b), and bottle 3 (c).

Appendix I Figures for Individual Bottles from pH Test on the DHM-1 Culture

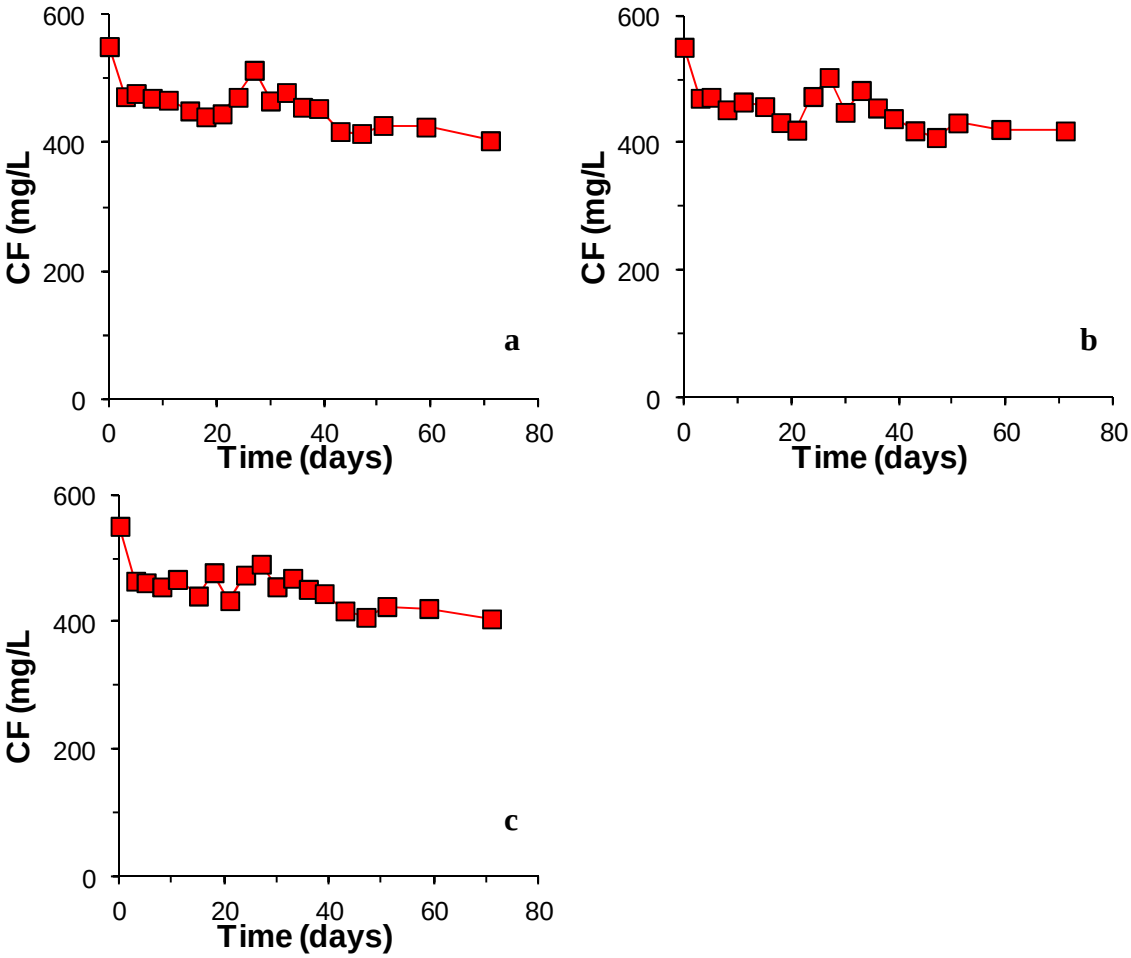

Figure I.1 CF transformation by DHM-1 when pH is 5.0 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

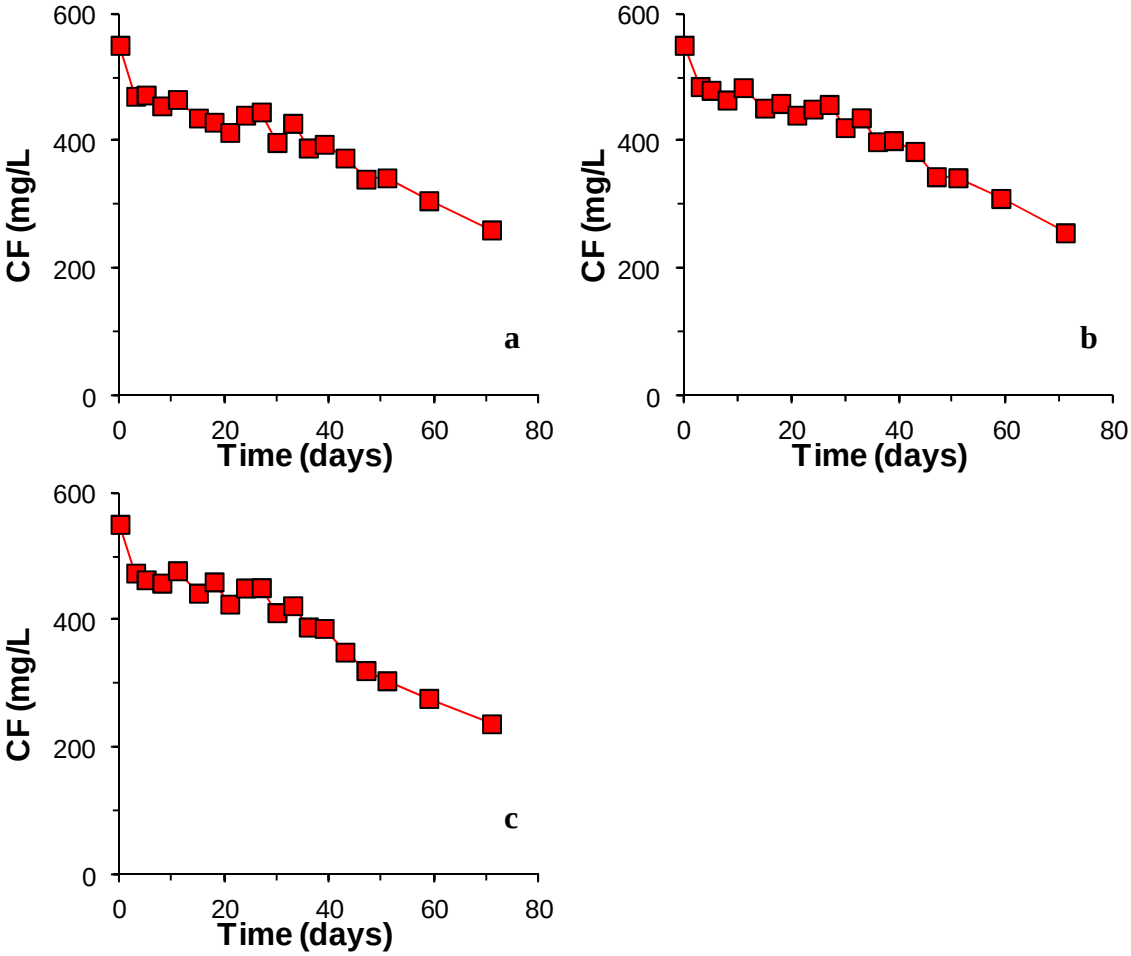

Figure I.2 CF transformation by DHM-1 when pH is 5.5 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

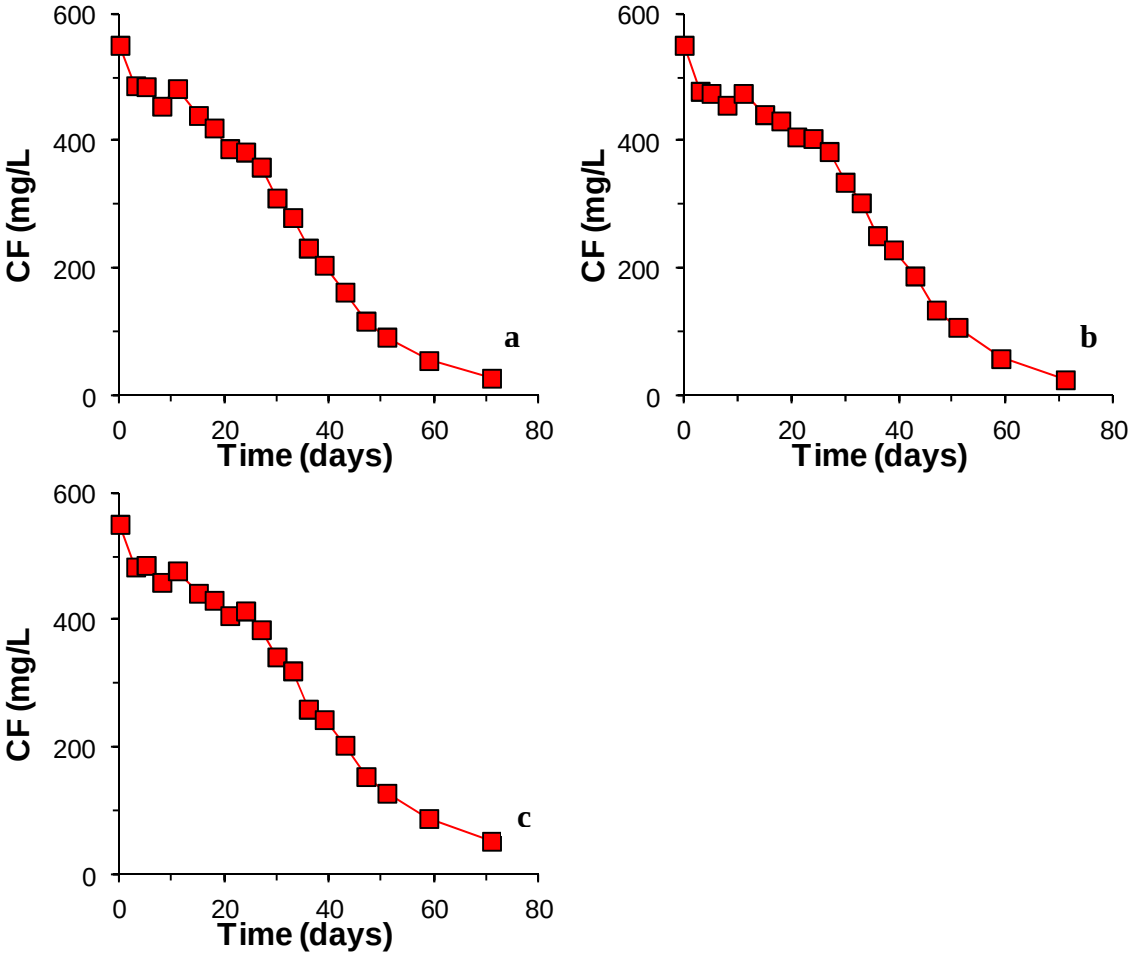

Figure I.3 CF transformation by DHM-1 when pH is 6.0 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

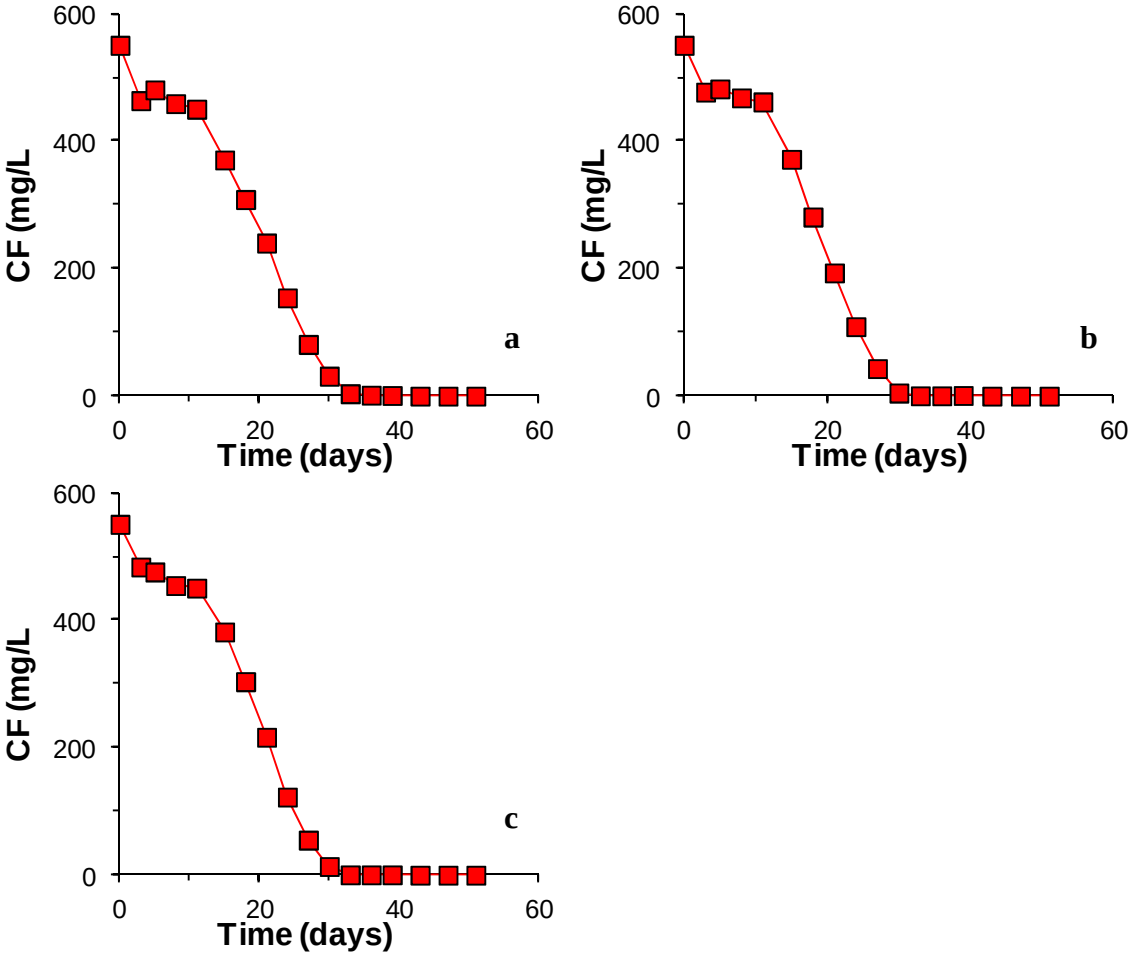

Figure I.4 CF transformation by DHM-1 when pH is 6.5 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

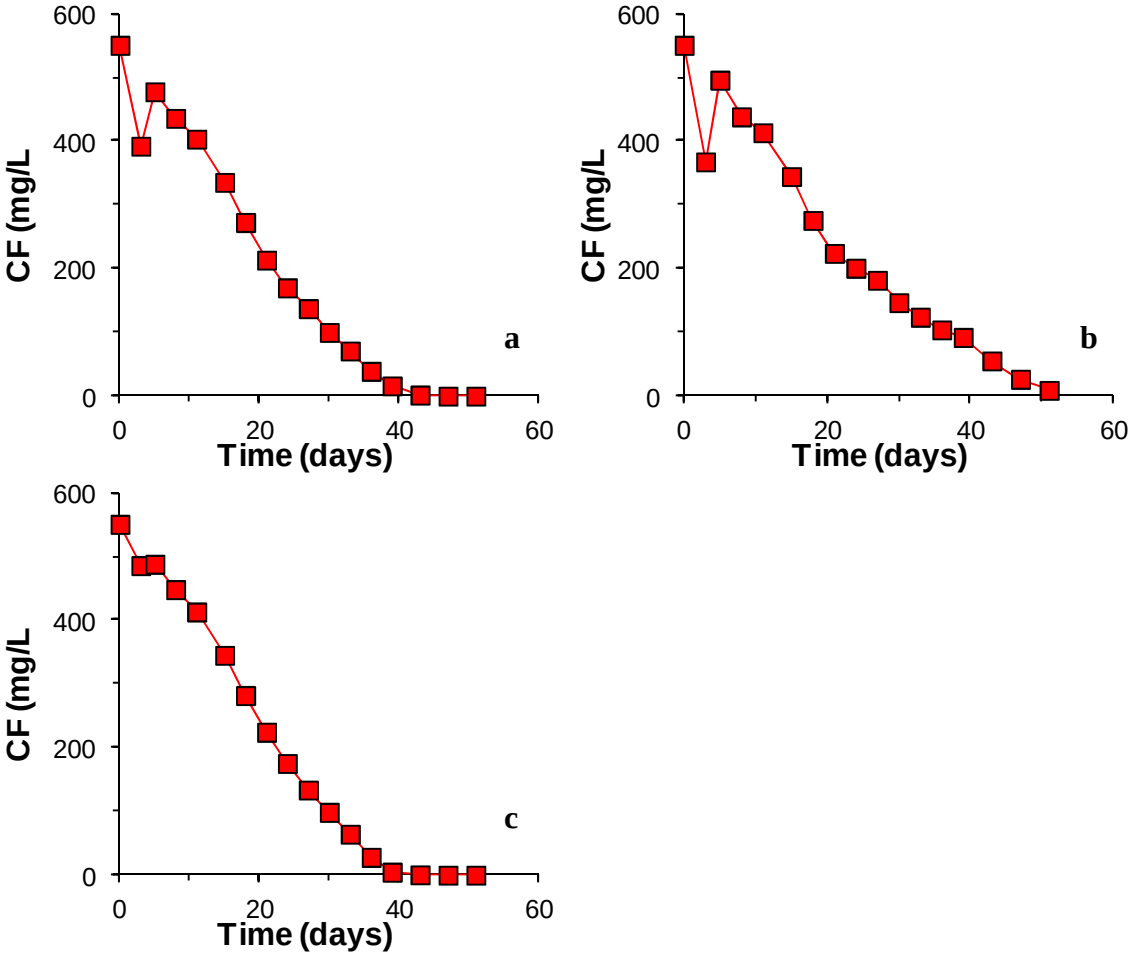

Figure I.5 CF transformation by DHM-1 when pH is 7.0 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

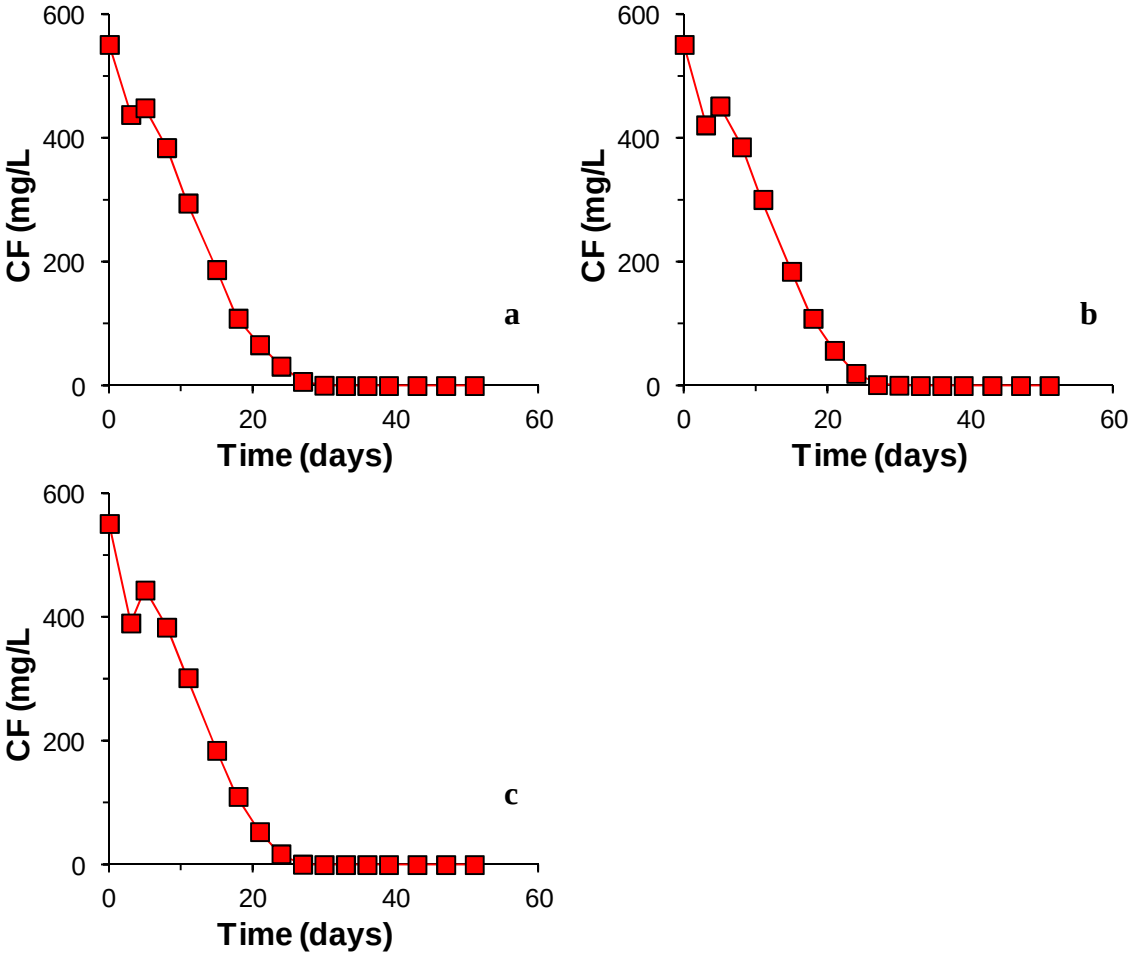

Figure I.6 CF transformation by DHM-1 when pH is 7.5 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

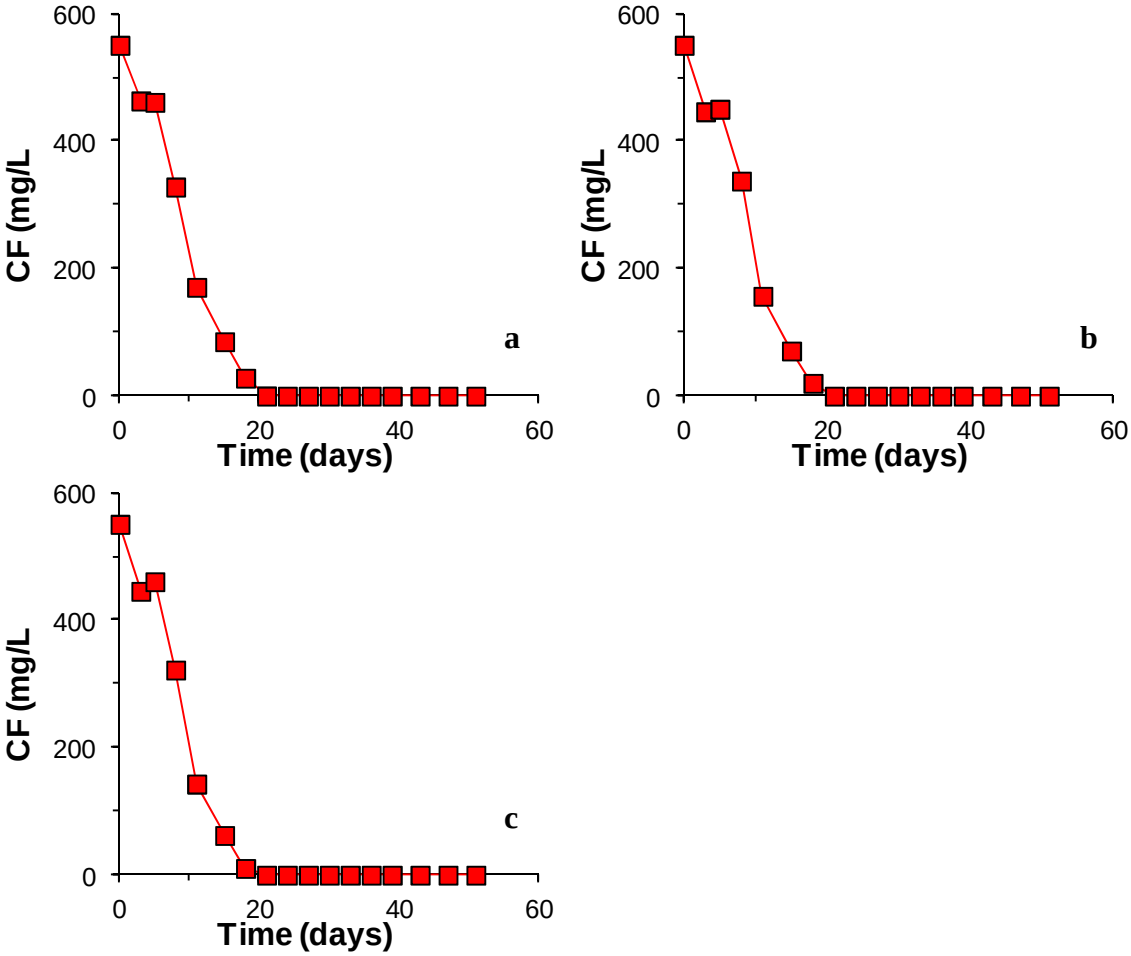

Figure I.7 CF transformation by DHM-1 when pH is 8.0 in bottle 1 (a), bottle 2 (b), and bottle 3 (c).

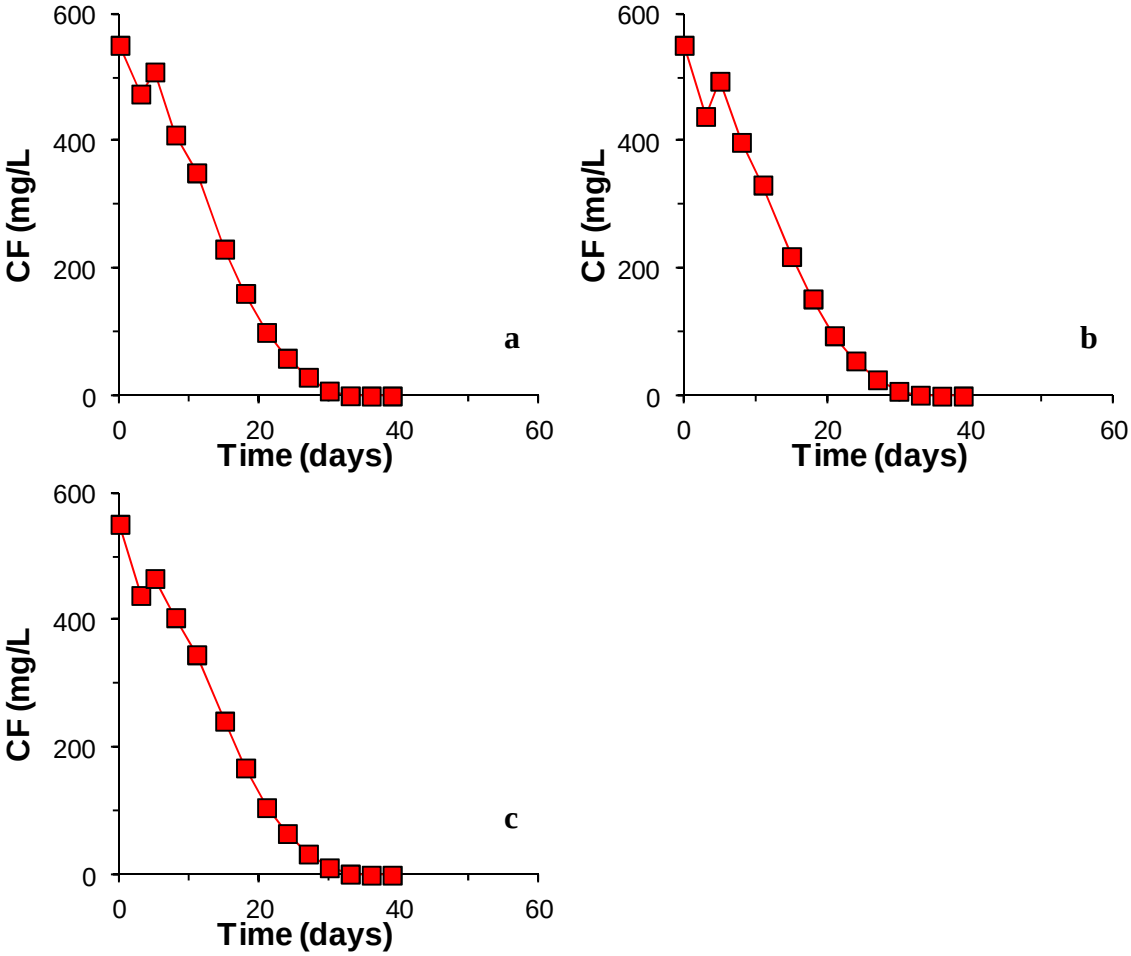

Figure I.8 CF transformation by DHM-1 in regular transfers bottle 1 (a), bottle 2 (b), and bottle 3 (c).

Appendix J Figures for Individual Microcosm Bottles from Site B

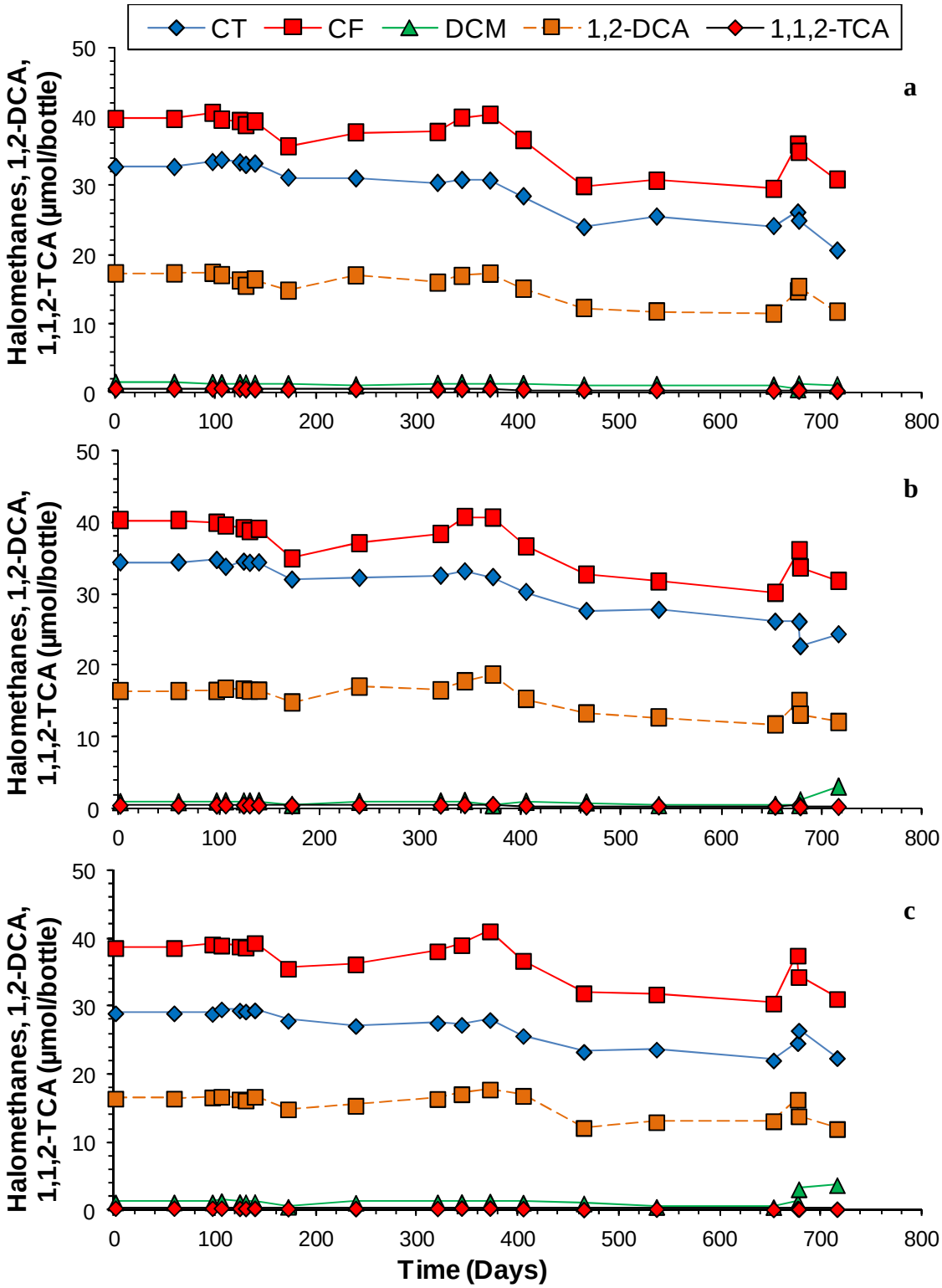

Figure J.1 Results for Site B, autoclaved control bottle 1 (a), bottle 2 (b), and bottle 3 (c).

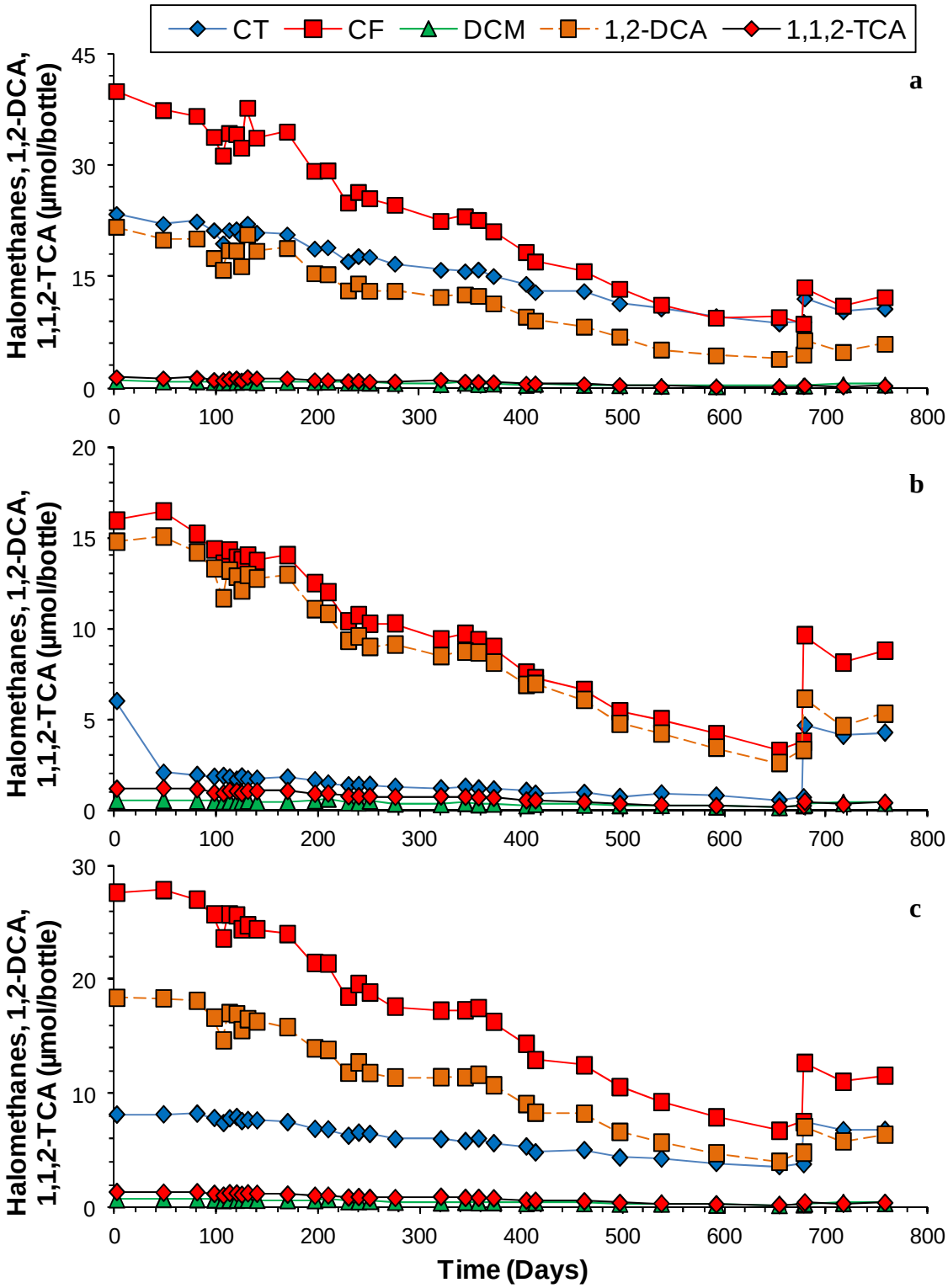

Figure J.2 Results for Site B, unamended treatment bottle 1 (a), bottle 2 (b), and bottle 3 (c).

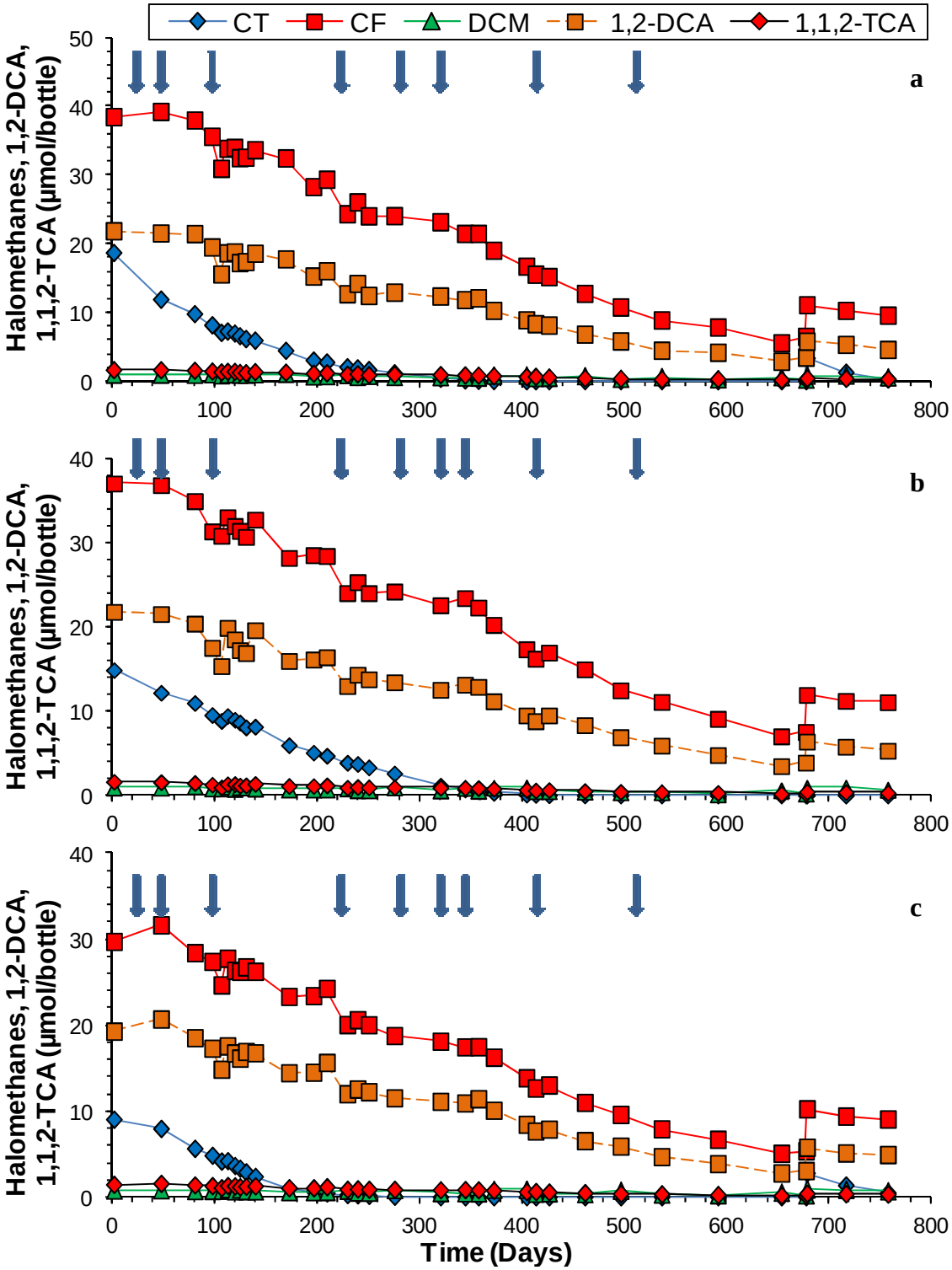

Figure J.3 Results for Site B, biostimulation with corn syrup bottle 1 (a), bottle 2 (b), and bottle 3 (c); ↓= addition of corn syrup.

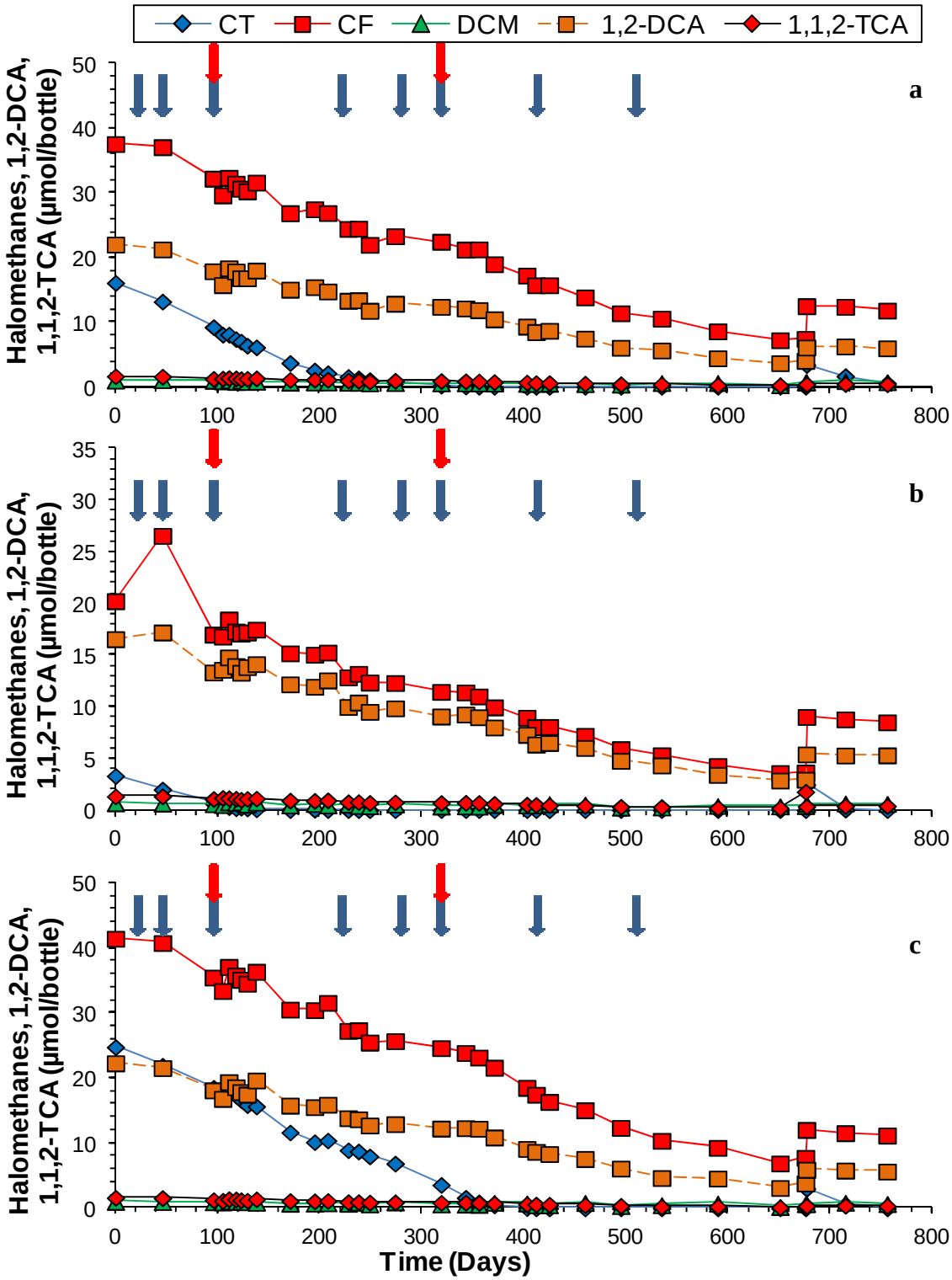

Figure J.4 Results for Site B, biostimulation with corn syrup + B_{12} bottle 1 (a), bottle 2 (b), and bottle 3 (c); ↓ = addition of corn syrup; ↓ = addition of B_{12}.

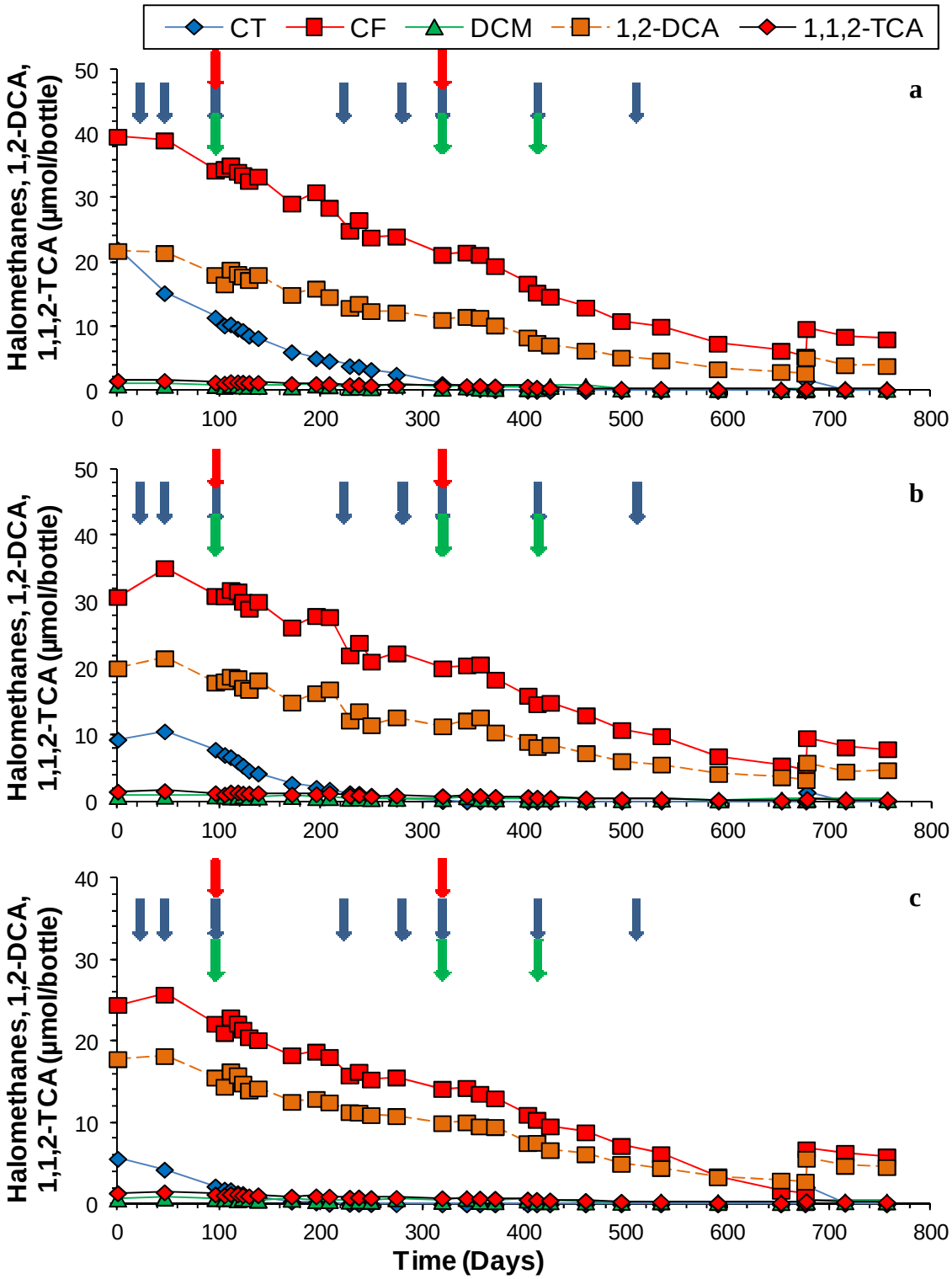

Figure J.5 Results for Site B, bioaugmentation bottle 1 (a), bottle 2 (b), and bottle 3 (c); ↓ = addition of corn syrup; ↓ = addition of B_{12}; ↓ = addition of DHM-1.

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